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Journal of Pharmaceutical and Biomedical Analysis 36 (2004) 743–749
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Direct determination of diclofenac in pharmaceutical formulations containing B vitamins by using UV spectrophotometry and partial least squares regression

Marcelo M. Sena[a], Zahra F. Chaudhry[b], Carol H. Collins[b], Ronei J. Poppi[a,*]

[a] *LAQQA, Laboratório de Quimiometria em Química Analítica, Departamento de Química Analítica, Instituto de Química, Universidade Estadual de Campinas, P.O. Box 6154, CEP 13083-862 Campinas, SP, Brazil*
[b] *LABCROM, Laboratório de Pesquisas em Cromatografia Líquida, Departamento de Química Analítica, Instituto de Química, Universidade Estadual de Campinas, P.O. Box 6154, CEP 13083-862 Campinas, SP, Brazil*

Received 19 March 2004; received in revised form 3 August 2004; accepted 5 August 2004
Available online 11 September 2004

Abstract

This work proposes a simple and rapid analytical procedure for determination of diclofenac (DCF) in the presence of B vitamins, based on UV measurements and partial least squares (PLS). The interference of thiamine (THI) and pyridoxine (PYR) were modelled using an experimental design constructed in the ranges of 10–50 μmol l^{-1} for DCF and THI and 15–75 μmol l^{-1} for PYR. The procedure was repeated at five different pH values (between 3 and 6) and the best results were observed at pH 5, presenting a root mean square error of prediction (RMSEP) of 0.80 μmol l^{-1} for DCF. The procedure was successfully applied to simultaneous determination of DCF, THI and PYR in synthetic mixtures and in a pharmaceutical formulation that contains a simple excipient (lactose). For determination of a more complex formulation that contains 15 different substances in the excipient, including some UV absorbing ones, the procedure was only able to determine DCF, since the excipient interferences disturbed THI and PYR predictions. Figures of merit, such as selectivity, analytical sensitivity, limit of detection and precision were determined for the DCF prediction model and the determinations were verified by an independent method, HPLC.
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Keywords: Diclofenac; Thiamine; Pyridoxine; Complex B vitamins; Multivariate calibration; PLS

1. Introduction

Diclofenac (DCF), 2-(2′,6′-dichloroanilino)-phenylacetic acid, is a nonsteroidal anti-inflammatory drug (NSAID) that has been used in the treatment of many rheumatic and nonrheumatic diseases [1]. It is employed in pharmaceutical formulations as the sodium or potassium salt, showing potent anti-inflammatory, analgesic and antipyretic properties, and being among the most effective inhibitors of prostaglandin synthesis. Reported side effects of DCF include gastrointestinal lesions, headache, dizziness, skin rashes, edema and hepatic and renal damage. B vitamins have been reported to potentiate the antinociceptive and analgesic effects of DCF and several papers have documented their contributions to reduce daily DCF dosage and shorten the treatment time [2–4]. Although some studies have questioned the antinociceptive efficacy of B vitamins concomitantly administered with DCF [5,6], these compounds have been commercialised together in a number of formulations in different countries in the last decade. These formulations usually contain similar quantities of DCF, thiamine (Vitamin B_1) and pyridoxine (Vitamin B_6) and 50–100 times less cyanocobalamin (Vitamin B_{12}). Thiamine (THI) is employed in pharmaceuticals either as the nitrate or the hydrochloride [7]. Pyridoxine (PYR) or pyridoxol is only one of the three similar compounds that are referred to as Vitamin B_6, the other two are pyridoxal and pyridoxamine. Only pyridoxine

* Corresponding author. Tel.: +55 19 37883126; fax: +55 19 37883023.
E-mail address: ronei@iqm.unicamp.br (R.J. Poppi).

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doi:10.1016/j.jpba.2004.08.001

hydrochloride, however, is used in pharmaceutical preparations [8].

Many analytical methods have been proposed for DCF determination in pharmaceutical formulations, the majority of them based on chromatographic or spectrophotometric procedures. Moreover, methods based on nuclear magnetic resonance spectroscopy (NMR) [9], differential scanning calorimetry (DSC) [10], potentiometry with an ion-selective electrode [11] and capillary electrophoresis [12,13] are also found in the literature. Among the chromatographic methods, HPLC [13–16], TLC [17], micellar chromatography [12,18] and LC-MS [19] can be cited and some of these are capable of determining DCF in the presence of some interferences, such as impurities [16,17], preservatives [16] and other active principles [14,19]. Many spectrophotometric methods have also been successfully used for DCF determination. Nevertheless, this direct determination can be hindered by the presence of interferences that absorb or fluoresce in the same region. Several authors have determined DCF indirectly by means of the formation of complexes that absorb in the visible [20,21], although this involves a time consuming solvent extraction step. An attempt to overcome this problem has been the use of flow injection analysis [22,23]. Other authors have determined DCF directly by measuring UV absorption [24,25] or emission fluorescence [26–28]. However, these methods are not able to determine DCF in the presence of THI and PYR, since their absorption spectra are strongly overlapped and PYR emission spectra also overlap that of DCF [26,29]. Only one determination of DCF in the presence of THI and PYR, based on solid phase UV absorptiometry, was found in the literature, but it has presented the drawback of demanding a solid phase extraction step [30]. The same authors of this last work [31] have also described an UV flow-through sensor based on solid phase retention for determination of DCF in the presence of some interferences (benzylic alcohol and paracetamol).

Since the last decade, the use of spectroscopic techniques combined with multivariate calibration can be considered a promising, faster, direct and relatively less expensive alternative for the determination of content in pharmaceutical formulations. So, research on this area is important, aiming at the future acceptance of these methods by the regulatory agencies. In this kind of situation, where the direct determination of an analyte is difficult due to the presence of one or several other constituents, instead of eliminating the interfering species, e.g. by a separation procedure, the use of multivariate calibration makes possible the quantification of these interferences along with the primary analyte. Partial least squares (PLS) [32,33] has been the most popular multivariate calibration method and is used for building regression models based on a latent variable decomposition relating a block of independent variables, x (spectra), to a block of dependent ones, y (concentrations or other properties). When the regression is carried out for each independent variable individually ($\mathbf{y}$ is a vector), it is called PLS1. When all independent variables are predicted simultaneously ($\mathbf{Y}$ is a matrix, whose number of columns is equal to the number of analytes), it is called PLS2. The combination of PLS and UV spectrophotometry has been used for simultaneous determination of several common active principles in pharmaceutical formulations, such as aspirin–caffeine–codeine [34], aspirin–paracetamol–caffeine [35], aspirin–ascorbic acid [36] and lidocaine and similar compounds [37]. Nevertheless, to the best of our knowledge, there is no published method to simultaneously determine DCF and the B vitamins THI and PYR that does not require a prior physical separation.

In this work, ternary mixtures of DCF, THI and PYR were studied by UV spectrophotometry and PLS. A reduced calibration matrix based on an experimental design was used to develop a simple, direct and rapid methodology for determination of DCF while modelling the interference of B vitamins in tablets and capsules. The results were verified by comparison with HPLC determinations of the same pharmaceutical formulations.

2. Experimental

2.1. Reagents

Diclofenac sodium was obtained from Galena (Campinas, Brazil). Thiamine and pyridoxine hydrochlorides were purchased from Sigma and Merck, respectively. Three stock solutions were prepared in 100 ml volumetric flasks: 6000 μmol l^{-1} PYR by dissolving 123.38 mg in water; 4000 μmol l^{-1} THI by dissolving 134.90 mg in water; and 4000 μmol l^{-1} DCF by dissolving 127.25 mg in methanol (Tedia)–water (50:50, v/v). Five intermediate solutions of each analyte were prepared from the stock solutions, in the following concentration values: 1000, 800, 600, 400 and 200 μmol l^{-1} for DCF (methanol–water, 50:50, v/v) and THI, and 1500, 1200, 900, 600 and 300 μmol l^{-1} for PYR. These solutions were stored at 4 °C in the dark and were observed to be stable for at least 3 months. The working standard solutions were prepared daily (see Section 2.3). Five buffer solutions (0.1 mol l^{-1}) were prepared in 250 ml volumetric flasks, one from H_3PO_4 (Sigma)/KH_2PO_4 (Merck), three from KH_2PO_4 and one from KH_2PO_4/K_2HPO_4 (Synth, Brazil). Their pH were adjusted with H_3PO_4 or KOH (Synth) at 3.00, 4.00, 4.50, 5.00 and 6.00, respectively. Deionised water obtained from a Millipore Milli-Q apparatus was used throughout.

2.2. Apparatus and software

The pH values were measured on a Corning pH/ion analyzer, model 350, previously calibrated with standard buffer solutions (4.00 and 7.00). An Agilent 8453 UV–vis diode-array spectrophotometer, equipped with a Peltier device, Agilent 89090A, for temperature control, was used and the Agilent UV–visible ChemStation software was utilised for data acquisition. All measurements were carried out at 20 °C,

Table 1
2^3 + 1 experimental design for the calibration set

Analyte/solution	1	2	3	4	5	6	7	8	9
DCF	+	+	+	−	−	−	+	−	∼
THI	+	+	−	+	−	+	−	−	∼
PYR	+	−	+	+	+	−	−	−	∼

Level (+): DCF 50.0 μmol l^{-1}, THI 50.0 μmol l^{-1}, PYR 75.0 μmol l^{-1}.
Level (−): DCF 10.0 μmol l^{-1}, THI 10.0 μmol l^{-1}, PYR 15.0 μmol l^{-1}.
Level (∼): DCF 30.0 μmol l^{-1}, THI 30.0 μmol l^{-1}, PYR 45.0 μmol l^{-1}.

in a quartz cuvette of 1.00 cm optical path. An ultrasonic bath was employed for sample extraction. The data were handled using MATLAB software, 6.1 version (The MathWorks, Natick, USA). PLS routine came from "PLS Toolbox", 2.0 version (Eigenvector Technologies, Manson, USA).

2.3. Procedure

2.3.1. Calibration set and synthetic mixtures

The calibration set was constructed according to a 2^3 + 1 (three factors at two-level plus one central point) experimental design (Table 1). The DCF and THI solutions were in the 10–50 μmol l^{-1} range and the PYR solutions were in the 15–75 μmol l^{-1} range. These ranges were selected around the values expected for the final concentrations of the analysed samples, after the dilution of the stock solutions. The different molar concentration range for PYR was chosen because of its lower molecular weight (204 g mol^{-1}) in relation to DCF (318 g mol^{-1}) and THI (337 g mol^{-1}), tacking into account that the mass content of the three substances was the same in the analysed formulations. Three points of the calibration set, the two extremes (+ and −) and the central point (∼), were determined in triplicate to estimate the mean precision of the method. The synthetic mixtures used to validate the model were planed according a 2^3 experimental design similar to the calibration set (without a central point). For this validation set, the level (+) was 40.0 μmol l^{-1} for DCF and THI and 60.0 μmol l^{-1} for PYR, and the level (−) was 20.0 μmol l^{-1} for DCF and THI and 30.0 μmol l^{-1} for PYR. Twenty-three standard solutions (calibration and validation sets) were prepared in 10 ml volumetric flasks by the addition of 500 μl of each intermediate solution of analyte and 5.00 ml of the respective buffer solution at each pH, using deionised water to complete the volumes. This procedure was repeated for all the pH sets. Although intermediate DCF solutions were prepared in 50% methanol/water, the standard solutions were 20 times diluted. Therefore, the final methanol content was 2.5% and the approximation that the pH values were the same as in a pure water media was used. The spectra of these solutions were scanned from 220 to 360 nm (1 nm steps). Solutions prepared in the same way as the mixtures, but containing none of the analytes, were used as the blanks for each pH set. Each blank solution was also measured 15 times aiming at estimating instrumental noise for determination of figures of merit. Spectra of pure DCF, THI and PYR solutions were also recorded at each pH value.

2.3.2. Determination of pharmaceutical samples

Two different pharmaceutical formulations available in Brazil, containing DCF and B complex vitamins, were acquired in local drugstores. They present the following compositions per capsule/tablet:

- Formulation 1 (capsules): 50 mg of sodium diclofenac, 50 mg of thiamine hydrochloride, 50 mg of pyridoxine hydrochloride, 1 mg of cyanocobalamin and excipient (lactose).
- Formulation 2 (tablets): 50 mg of sodium diclofenac, 50 mg of thiamine mononitrate, 50 mg of pyridoxine hydrochloride, 1 mg of cyanocobalamin, talc, magnesium stearate, lactose, cellulose, sodium carboxymethylcellulose, colloidal silicon dioxide, Eudragit RL 30D, macrogol, titanium dioxide, dimethicone, triethylcitrate, methylparaben, propylparaben, povidone and red dye FD&C no. 6.

Ten tablets and the powder from 10 capsules were weighed individually to obtain representative average weights. The tablets were finely powdered and mixed. The powder from the capsules were also mixed. A mass corresponding to one capsule or tablet for each formulation was accurately weighed and dissolved in 250 ml of methanol/water (50:50, v/v), in a volumetric flask. Dissolution was carried out with the aid of an ultrasonic bath (15 min). An aliquot of 500 μl of each sample was added to 10 ml volumetric flasks together with 5.00 ml of the respective buffer solution at the specified pH and deionised water was used to complete the volume. The spectra were obtained using the same conditions already described. All these determinations were performed in triplicate.

2.4. Chromatographic analysis

The verification by HPLC was carried out with a Shimadzu liquid chromatograph, consisting of an LC 10 AD pump and an SPD 10AV UV detector with a 5 μl injection valve (Rheodyne). Chrom Perfect for Windows software, version 3.52 (Justice Innovations Inc.) was used for data acquisition. The analytical column (150 mm × 3.9 mm) utilised was developed by LABCROM and was packed with 5 μm Rainin silica having thermally immobilised coating of poly(methyl octadecylsiloxane) [38]. The mobile phase was methanol/water (70:30, v/v) adjusted at pH 3.8 with phosphoric acid. A flow rate of 1.0 ml min^{-1} and detection at 275 nm were used. All the injections were repeated three times and as in the spectrophotometric/chemometric procedure, sample determinations were performed in triplicate.

3. Results and discussion

3.1. DCF, THI and PYR UV absorption spectra

The pK_a values found in the literature for the studied drugs are: 4.84 for DCF [27], 4.75 for THI [39] and 5.00 for PYR

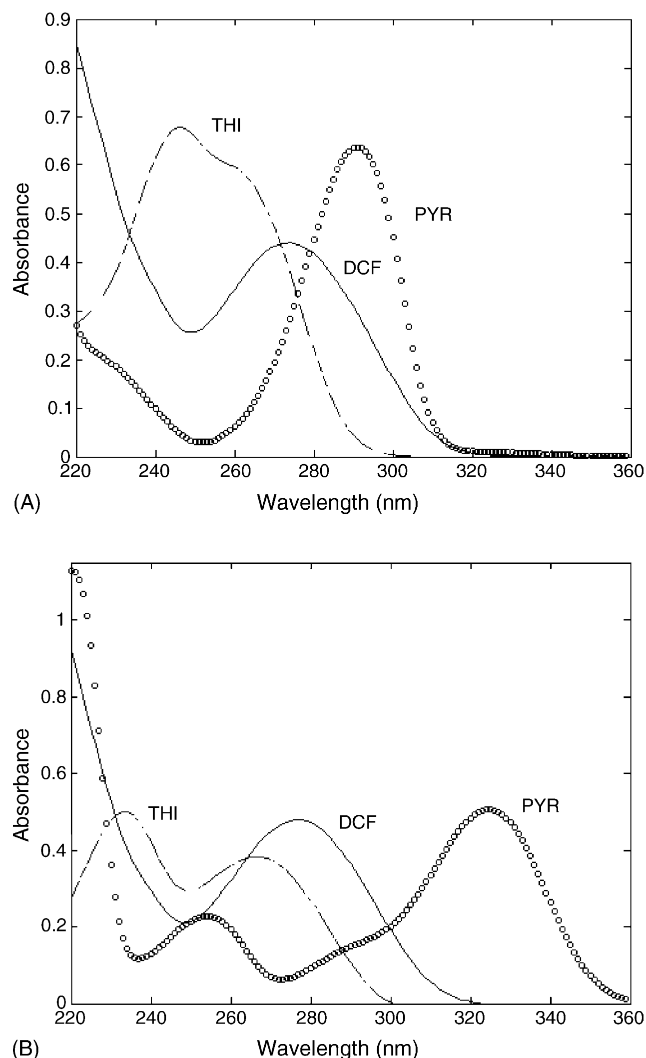

Fig. 1. UV absorption spectra of DCF, THI and PYR: (A) pH = 3.00; (B) pH = 6.00. [DCF] = [THI] = 50.0 μmol l^{-1} and [PYR] = 75.0 μmol l^{-1}.

[8]. Taking into account these pK_a values, it was decided to carry out this study in the pH range from 3 to 6. Fig. 1A displays the UV spectra for aqueous solutions of DCF, THI and PYR obtained at pH 3.00, where only the acidic forms of these analytes are present; Fig. 1B displays the UV spectra obtained at pH 6.00, representing the pure basic forms of these same analytes. The spectra of both acidic and basic forms of DCF are very similar, showing a small shift of λ_{max} from 274 to 277 nm. This spectral similarity can be attributed to the occurrence of ionisation in the carboxylic site far from the chromophore moiety (aromatic rings). As can be observed in Fig. 1, there is a strong overlap among the spectra, in both the acidic and the basic media. Although PYR could be univariately determined in basic medium at 325 nm (Fig. 1B), the DCF spectra remain strongly overlapped by the other two, which prevents the use of univariate calibration without a previous separation step.

3.2. Calibration and validation of PLS models

Multivariate calibration methods demand a suitable experimental design of the standards belonging to the calibration set in order to have good predictions. The calibration set was built using nine solutions according to the experimental design shown in Table 1. Another eight solutions were used as the validation set, according to a second experimental design, whose range was included in the calibration design. Although this two-level plus one central point calibration design does not have the four or five concentration levels usually required for each compound [33], the number of levels depends strongly on the nature of the system under calibration. For more complex matrices, e.g., soil samples analyzed by IR, this design would certainly be insufficient, but for a simple matrix, such as in this case (synthetic aqueous solutions), it is appropriate as is demonstrated by the results and other successful applications found in the literature [34,36,40–43].

PLS models were constructed for each pH data set. The number of latent variables chosen, three for all the models, was obtained from a leave one out cross-validation procedure. The root mean square error of prediction (RMSEP) of the validation sets was the parameter employed for comparison among the models. RMSEP is given by

$$\text{RMSEP} = \sqrt{\frac{\sum (y_r - \hat{y}_p)^2}{n}} \quad (1)$$

where y_r is the standard (real) value and $\hat{y}_p$ the value predicted by the model.

As mentioned in the introduction, the strategy of modelling the interferences of THI and PYR along with the primary analyte DCF was adopted, based on an experimental design, and it was possible to simultaneously determine these three analytes using PLS2. Table 2 shows the RMSEP values for the calibration models at each pH using three latent variables. The best PLS2 model for the DCF prediction was obtained at pH 5.00. Nevertheless, better results were obtained for THI and PYR predictions at pH 4.00. As our primary interest was DCF determination, PLS2 at pH 5.00 was the chosen model. The predictions for synthetic mixtures from the validation set obtained with this model are shown in Table 3, in order to demonstrate the accuracy of the proposed method. All the errors of prediction were below 5% (the great majority were below 2%). PLS1 models (only DCF prediction) provided results practically identical to the PLS2 ones for this compound (the difference is in the third decimal digit).

3.3. Analytical figures of merit

The determination of figures of merit (FOM) is an important requisite for the validation of this kind of chemometric/spectrophotometric methods, aiming at their possible acceptance by the regulatory agencies in the future. FOM, such as sensitivity, selectivity and precision, can be estimated and used to compare analytical methods. When

Table 2
Root mean square errors of prediction (RMSEP) between the real and the predicted values obtained for eight synthetic mixtures (validation set), for PLS2 models at each pH studied

Analyte	pH 3.00 (μmol l^{-1})	pH 4.00 (μmol l^{-1})	pH 4.50 (μmol l^{-1})	pH 5.00 (μmol l^{-1})	pH 6.00 (μmol l^{-1})
DCF	3.44	3.05	1.56	0.80	3.08
THI	0.69	0.24	0.37	0.38	0.98
PYR	1.42	0.35	0.53	0.70	1.37

Table 3
Simultaneous determination of DCF, THI and PYR in eight different synthetic mixtures (validation set) using PLS2 model at pH 5.00

Amount added (μmol l^{-1})			Amount predicted (μmol l^{-1})			Error (%)		
DCF	THI	PYR	DCF	THI	PYR	DCF	THI	PYR
40.0	40.0	60.0	40.0	39.6	59.8	0.0	−1.0	−0.3
40.0	40.0	30.0	41.9	40.3	30.6	4.8	0.8	2.0
40.0	20.0	60.0	40.7	20.2	61.1	1.8	1.0	1.8
20.0	40.0	60.0	20.1	39.5	59.9	0.5	−1.3	−0.2
20.0	20.0	60.0	20.6	20.3	61.2	3.0	1.5	2.0
20.0	40.0	30.0	20.6	40.7	30.7	3.0	1.8	2.3
40.0	20.0	30.0	40.0	20.1	30.4	0.0	0.5	1.3
20.0	20.0	30.0	20.4	20.2	30.5	2.0	1.0	1.7

expressing FOM for multivariate calibration methods, the part of the signal that relates uniquely to the analyte of interest is more important than the total signal. This unique signal is termed net analyte signal (NAS) and is defined as the part of the signal that is orthogonal to the signal of the interferences present in the sample [44]. The **NAS** is a vector containing the values for each sample and can be related to the regression vector, **b**, from an inverse calibration model, such as PLS, by the following equation [45]:

$$\|\mathbf{NAS}\|_2 = \frac{1}{\|\mathbf{b}\|_2} \quad (2)$$

where the symbol $\| \|_2$ means the Euclidian norm of a vector. FOM can subsequently be calculated as functions of the **NAS** (or the regression vector) [45]. Sensitivity (SEN) is estimated as the **NAS** at unit concentration, according to Eq. (3). Selectivity (SEL) is a measure, ranging from 0 to 1, of how unique the spectrum of the analyte is, compared with the other species. SEL is estimated as the ratio between SEN and the total signal (x), according to Eq. (4):

$$\mathrm{SEN} = \|\mathbf{NAS}\|_2 \quad (3)$$

$$\mathrm{SEL} = \frac{\|\mathbf{NAS}\|_2}{\|\mathbf{x}\|_2} \quad (4)$$

A more informative FOM is the analytical sensitivity (γ), which is defined, in analogy with univariate calibration [46], as the ratio between SEN and the instrumental noise (ε), according to Eq. (5). The term $\|\varepsilon\|_2$ was estimated from fifteen replicates of the blank measurement:

$$\gamma = \frac{\mathrm{SEN}}{\|\varepsilon\|_2} \quad (5)$$

with the inverse of γ (γ^{-1}), it is possible to establish a minimum concentration difference that is discernible by the analytical method in the absence of experimental error, independent of the specific technique employed.

The limit of detection (LOD), the smallest concentration of analyte in the test sample that can be reliably distinguished from zero, was calculated as follows:

$$\mathrm{LOD} = \frac{3\|\varepsilon\|_2}{\|\mathbf{NAS}\|_2} \quad (6)$$

Precision represents the degree of scatter between a series of measurements for the same sample under prescribed conditions. In this work, we estimated the mean precision according to Eq. (7):

$$\text{Mean precision} = \sqrt{\frac{\sum_i^n \sum_j^m (\hat{y}_{ij} - \bar{\hat{y}}_i)^2}{n(m-1)}} \quad (7)$$

where n is the number of replicated samples and m the number of replicates for each sample. Three samples (two extremes plus the mid point) from the calibration set were triplicated ($n = m = 3$) and their predictions by the model were used for estimating the mean precision.

Table 4 presents FOM estimates for DCF determination with the PLS2/pH 5 model. The estimated LOD and γ are equivalent to 0.1 μg ml^{-1} and 25 ml μg^{-1}, respectively. This γ estimate means that the proposed method is able to discern a difference of 0.1 μmol l^{-1}, in the absence of experimental

Table 4
Analytical figures of merit for DCF determination with PLS2 model at pH 5.00

Figures of merit	Estimate
SEL	0.15
SEN	0.03
γ (l μmol^{-1})	8.0
LOD (μmol l^{-1})	0.4
Mean precision (μmol l^{-1})	0.4

Table 5
Determination of DCF in two pharmaceutical formulations also containing B vitamins by the proposed chemometric/spectrophotometric method and HPLC

Formulation	Label claim (mg)	Proposed method (mg)[a]	Verification method (mg)[a]
#1	50	47.8 ± 0.7	47.2 ± 3.5
#2	50	52.2 ± 1.9	51.0 ± 1.0

[a] Mean values and standard deviations of three determinations.

error. This analytical sensitivity, together with the estimated precision, are indications of the good quality of the model.

3.4. Analysis of real samples and HPLC verification

Firstly, PLS2/pH5, the best calibration model, was applied to simultaneous determination of DCF, THI and PYR in two different pharmaceutical formulations. These formulations have the same amounts of active substances, but different excipient compositions. The excipient of formulation 1 (capsules) contains only one substance, lactose, while formulation 2 (tablets) has a more complex excipient mixture containing 15 substances. The predictions of THI and PYR for formulation 1 are 53.1 ± 0.3 and 51.9 ± 1.5 mg (mean values and standard deviations of three determinations), respectively. These results are in agreement with those specified by the manufacturers (label claims of 50 mg for each one), taking into account the tolerance level of ±10% established in the US Pharmacopoeia [15] for this type of drug. On the other hand, it was only possible to determine DCF in formulation 2 (differences inferior to 5%), because the predictions for THI and PYR were more than 10% higher than those claimed on the label. These results can be explained by the presence of substances in the excipient of formulation 2, such as methylparaben, propylparaben and possibly others, which absorb in the same spectral regions, as do the analytes. Both the parabens present a strong, broad band centred on around 260 nm (these spectra were obtained experimentally). As these substances were not presented in the calibration set, they were not modelled and their interference can be considered responsible for the prediction errors. Another possible interference would be cyanocobalamin (Vitamin B_{12}), which was not present in the calibration set, but is contained in both formulations in an amount 50 times lower than the other active substances. However, an absorption peak at 360 nm due to cyanocobalamin [47] was not observed and this interference can be considered insignificant. An alternative for the simultaneous determination of the three analytes in formulation 2 would be to conduct a systematic study to identify all the excipient substances that significantly interfere in the predictions of the B vitamins and, in sequence, to incorporate these substances in the PLS model according to a new experimental design. However, in spite of the problems in quantifying the B vitamins, accurate results for DCF predictions were obtained and verified by an HPLC analysis.

DCF predictions obtained with PLS2/pH5 model are shown in Table 5, together with the results from an HPLC analysis used to verify the method. The HPLC determinations of DCF were also carried out in triplicate, using samples prepared independently of the ones utilised in the spectrophotometric analyses. A t-test with four degrees of freedom was used to compare the results of the spectrophotometric and the chromatographic methods and the estimates were considered identical at 95% of confidence level for both formulations (t = 0.273 for formulation 1 and t = 0.559 for formulation 2, both considerably less than the tabulated value, t = 2.776).

4. Conclusions

The proposed PLS spectrophotometric procedure was able to determine diclofenac in the presence of strongly overlapped interferences of B vitamins and other excipient substances. A relatively small calibration set was required based on the experimental design. Precise and accurate results were obtained based on the estimation of figures of merit, and verified by HPLC. Thus, this method may be posed as a possible alternative in the quality control analysis of this pharmaceutical.

For the determination of diclofenac in pharmaceutical formulations that contain simple excipients, such as formulation 1, the method is able to simultaneously determine diclofenac and the B vitamins. For a specific more complex formulation that contains others spectrophotometrically active substances in the excipients, such as formulation 2, the method was able to accurately determine only diclofenac.

Acknowledgments

MMS thanks CNPq for a fellowship.

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