DETERMINATION OF THE ENANTIOMERIC RATIO OF WARFARIN IN TABLETS BY MULTIVARIATE CALIBRATION AND MODELING OF SPECTROSCOPIC DATA

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Abstract

Considering the constant need to develop high performance, selective and robust analytical chiral methods, the aim of the present work was the determination of enantiomeric excess of warfarin by multivariate calibration and modeling of spectral data.

Chromatographic and electrophoretic chiral separations have proven their value, offering good selectivity and low limits of quantification; however they may also require lengthy and expensive method development and prolonged analysis times.

In this paper we have developed a simple method for the determination of the chiral purity of warfarin from tablets by chemometric spectroscopic calibration, using t-butyl-calix[6]arene as chiral selector. The obtained results were confirmed also by chiral high-performance liquid chromatography.

Rezumat

Considerând necesitatea constantă de a dezvolta metode analitice chirale de înaltă performanţă, selective şi robuste, scopul prezentei lucrări a fost determinarea excesului enantiomeric al warfarinei prin calibrare multivariată şi modelare a datelor spectrale.

Separările chirale cromatografice şi electroforetice şi-au dovedit utilitatea, oferind o selectivitate bună şi limite mici de cuantificare; totuşi dezvoltarea acestor metode pot fi îndelungată şi costisitoare, ceea ce poate conduce la timpi lungi de analiză.

În prezenta lucrare s-a dezvoltat o metodă chemometrică simplă pentru determinarea purităţii optice a warfarinei din tablete, utilizând t-butil-calix[6]arena ca selector chiral. Rezultatele obţinute au fost, de asemenea, confirmate şi prin cromatografie de lichide de înaltă performaţă chirală.

Keywords: warfarin, t-butyl-calix[n]arene, multivariate data analysis, UV spectroscopy

Introduction

Chiral drugs represent more than a half of the total marketed drugs [1] and the majority among them are commercialized as single enantiomers, the chiral switch being a present phenomenon in chiral drug industry.

Oral anticoagulants are drugs with a high risk of administration because of their low therapeutic index, showing also plasmatic concentration variability from one patient to another.

Warfarin [4-hydroxy-3-(3-oxo-1-phenylbutyl)-coumarin] or coumafene (Figure 1) is a synthetic oral anticoagulant, acting as an anti-vitamin K agent, widely used in the prevention and treatment of thromboembolism, which can occur in venous thrombosis, post-myocardial infarction, stroke or mechanical valve replacement [2].

They are metabolized by different isoenzymes of cytochrome P450, belonging especially to the 2C9 family. These many routes of metabolism lead to differences in their pharmacodynamics, the S-warfarin being five times more active than the R-enantiomer and also, facilitating a various number of warfarin-drug interactions, which are difficult to control [2].

Several articles dealing with the enantioseparation of warfarin have been recently published [3-6]. Mostly they include liquid chromatographic [3,4] or electrophoretic techniques [5,6]. These chiral separation techniques have proven their value, offering good selectivity and low limits of quantification; however they may also require lengthy and expensive method development and prolonged analysis times.

Simultaneous determination of components in a multicomponent drug formulation without separation could be a difficult task, especially when characteristics of these components from the analytical point resemble closely in addition to the presence of other pharmaceutical excipients. Recently, spectroscopic calibration as part of multivariate chemometric methods for the analysis of multicomponent systems, including chiral analysis, have been reported mostly due to the advent of rapid scanning spectrophotometers, fast and affordable computers and user-friendly chemometry software.

Spectroscopic calibration is the process of using mathematical algorithms to relate the measured spectroscopic properties of a set of samples to the chemical composition (and sometimes other properties) of those samples, with the aim of predicting the composition of new samples based on a single measurement of the spectrum. In contrast to separation techniques, there is no need to have a chiral separation to know the enantiomeric purity of the sample. Also compared to other methods, there is no need to use polarized light, because the two enantiomers form with the chiral selector diastereomeric complexes that present slightly different physical and spectral properties.

Materials and Methods

Sample preparation and spectroscopic determinations

Warfarin (+/-) and its enantiomer, (R)-(+)-warfarin were purchased from Sigma-Aldrich®, both at analytical standard degrees.

Warfarin containing tablets used for the method validation were Warfarin Orion® 3mg tablets from Orion Corporation (Finland).

Tert-butyl-calix[6]arene (C6) was employed as chiral selector and it was kindly donated by the group of J. Popovici from the Raluca Ripan Institute of Chemistry, Cluj-Napoca. Chloroform was purchased from Merck (Germany) and used as it is throughout the experiments.

A stock solution of 3.15 mg/mL of C6 in chloroform was used for the preparation of all the training and validation sets, maintaining a fixed concentration of 3.24mM of the chiral selector. Training and validation sets of known warfarin enantiomeric ratios were prepared with the aid of stock solutions of (+/-)-warfarin and (R)-(+)-warfarin in chloroform (~3.3mM total warfarin).

As real sample, an accurately weighed portion of the finely powdered tablets corresponding to 1 mg of pure warfarin was extracted by sonication in 1 mL chloroform. From the obtained supernatant after centrifugation at 5000 rpm for 15 minutes the extracted total warfarin concentration was spectrophotometrically determined at 307 nm using a previously assessed linear regression (Absorbance = 0.0293 x (μg/mL) – 0.0152, r^2=0.9993) on a modular fiber optics AvaSpec spectrometer (Avantes, Spain) using 1.0 cm pathway quartz cell. By adding the proper aliquot of the chiral selector's stock solution the solution was made up with chloroform in a 5 mL volumetric flask resulting 3.3mM total warfarin and 3.24 mM C6.

All samples, including pure chloroform were applied as spots (1 μL, speed of 3 s/μL) on a 10 x 5 cm Silicagel HPTLC plate (Merck, Germany) with the aid of a semi-automated AS-30 Desaga (Germany) TLC sample applicator. After plate drying, the reflectance spectra (Figure 2) were recorded with a CD60 Desaga Photodensitometer (Germany), on the range of 200-400 nm, 1nm resolution, 64 readings/point, scanning window 0.2 x 1 mm, considering as reference the spot of chloroform.

Chiral HPLC

For the confirmation of the obtained results on real samples, chiral separation using high-performance liquid chromatography was employed. Polysaccharide-based, Lux Amylose-2 (5μm, 250 x 4.6 mm, Phenomenex, USA) chiral stationary phase column was used on a Agilent Series 1200 HPLC system equipped with a DAD detector. The mobile phase consisted

of acetonitrile with 0.1% (v/v) dietylamine and 0.1% (v/v) trifluoracetic acid delivered at 1 mL/min at 35°C. The injected sample volume was 5 μL.

Multivariate data analysis

Spectral data were subjected to partial least-squares (PLS) multivariate regression analysis using Simca-p+ software (Umetrics, Sweden). PLS regression was performed on the reflectance spectral data using full cross validation of the training set. PLS was used to develop a semi-empirical mathematical model that correlates spectral data over many wavelengths with the enantiomeric composition, expressed as the (R)-(+)-warfarin mole fraction (x_R). The process involves two steps: (a) the model is trained to predict x_R from the calibration set (samples with known enantiomeric composition), and (b) external model validation, in which a second, independent set of samples (validation set, also with known enantiomeric composition) is analysed over the same range of wavelengths in the same conditions. The predicted enantiomeric composition is then compared with the known reference values.

Results and Discussion

The training set consisted of six samples (x_R = 0.5, 0.6, 0.7, 0.8 0.9 and 1), whereas five were used as the calibration set (x_R = 0.55, 0.65, 0.75, 0.85 and 0.95) (Tables I and II).

Table I.

Warfarin training set parameters

	Y-block	X-block
Observation ID identifier	**Mole ratio of R-(+)-warfarin (x_R)**	**Total concentration of warfarin (mM)**
50%	0.5000	3.2757
60%	0.6016	3.2887
70%	0.7024	3.3017
80%	0.8023	3.3146
90%	0.9016	3.3276
100%	1.0000	3.3406

Due to practical issues concerning sample preparation the total warfarin concentration in both the training and validation set is allowed to vary within a limited range, however the total concentration of warfarin, along with the spectral data, was included in the model as an X variable. Warfarin has one chiral center and by different spectroscopic techniques (FT-IR, Raman) it has been proven to form 1:1 inclusion complexes with tert-butyl-calix[6]arene (spectra not shown). Therefore in all the training,

calibration or real samples containing warfarin a fixed concentration of 3.24 mM tert-butyl-calix[6]arene as chiral selector was added.

Spectral data analysis began with a principal component analysis (PCA) to detect outliers and other anomalies in the data. PCA of the mean-centered X-data gave a two-component model, which explained 87.4% of the variation (R^2X=0.874). Examining the loadings, the first component, accounting for more than 56%, captures the overall variation in the low and mid range of the spectra, whereas the loading of the second component describes mainly the spectral variation in the range of 250 – 310 nm. The PCA-X model, apart from identifying the outliers, can also be useful in giving the first hint of the spectral range loaded with the most useful information considering the chiral analysis. Continuing with the PLS modeling of the entire data set, as part of data pre-treatment, all the spectral data (Figure 2) recorded on the range of 200 – 400 nm were mean centered, whereas the total molar concentration of warfarin and the mole fraction of (R)-(+)-warfarin (x_R) was unit variance scaled and mean centered. Throughout the model optimization, in order to enhance the predictive power of the calibration model by eliminating variation in X that is unrelated to Y, the spectral data was further pre-processed prior to data analysis by the use of different spectral filters. The one that offered the best result turned out to be the standard normal variate (SNV) signal correction. In the PLS model, the X-block comprised in 202 variables (201 spectral data, plus the total concentration of warfarin) and the Y-block the mole fraction of R-(+)-warfarin (x_R).

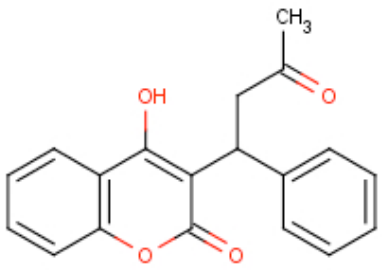

Figure 1. The structure of (+/-)-warfarin

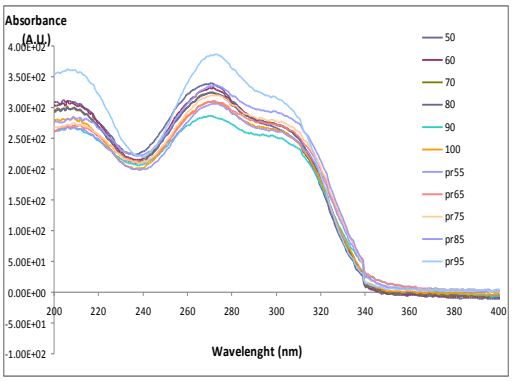

Figure 2. UV - reflectance spectra of training and validation set of varying mole fractions of R-(+)-warfarin in the presence of 3.24mM tert-butyl-calix[6]arene; 200-400 nm

The PLS modeling yielded a one-component model, which according to cross-validation gave an explained variation (goodness of fit) of R^2Y = 0.999 and the predicted Y-variation according to internal cross-validation (goodness of prediction) of $Q^2_{int}Y$ = 0.998. In order to obtain an estimate of the significance of the Q^2Y-value a response permutation was carried out, which consists in developing a number of parallel models based on fit to randomly re-ordered Y-data, and evaluates the real Q^2Y in the light of a distribution of Q^2Y-values of the re-ordered response data, giving a statement of the statistical significance of the estimated predictive power, ruling out any overfitted model (R^2Y= -0.12, Q^2Y= -0.448; Crit.: R^2Y<0.3-0.4, Q^2Y<0.05). Predictive validation by means of cross-validation and response permutation testing in many ways provides a reasonable first approximation of the predictive ability of the PLS model. However, a more demanding and rigorous way of testing predictive performance consists of computing predictions for an independent set of test observations (validation set). The results of the external validation of the obtained PLS model is presented in Table II. The very high value of goodness of prediction ($Q^2_{ext}Y$= 0.9995), the low Root Mean Square Error of Prediction (RMSEP = 0.0164363) and the good correlation (y=1.022x-0.03295, r^2=0.9995) between real and predicted mole fraction values of R-(+)-warfarin from the validation set (Figure 3) all prove the high analytical value of the obtained model.

The chemometric analysis of the real sample of warfarin based on the UV spectra of the chloroformic extract gave the expected results since the tablets of warfarin contain racemic warfarin, fact also confirmed by chiral HPLC separation (Table III).

Table II.

Results for the external validation of the PLS model

Mole ratio of R-(+)-warfarin (x_R)		ERROR OF PREDICTION (%)
REAL	PREDICTED	
0.5509	0.5734	4.08
0.6521	0.6652	2.02
0.7525	0.7699	2.32
0.8521	0.8686	1.94
0.9509	0.9606	1.02

Table III.

Mole fraction of R-warfarin enantiomer in real samples

Warfarin Orion® 3mg tablets	Mole ratio of R-(+)-warfarin (x_R)		ERROR (%)
	REAL	OBTAINED	
Chemometry	0.5000	0.5055*	1.10
Chiral HPLC	0.5000	0.5022	0.44

* n=2, RSD = 1.25%

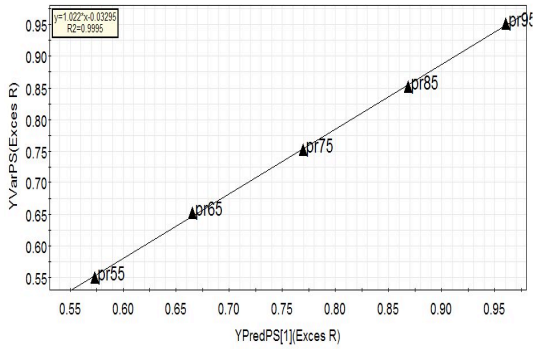

Figure 3. Predicted *versus* real values of mole fraction of R-(+)-warfarin of the used independent validation set

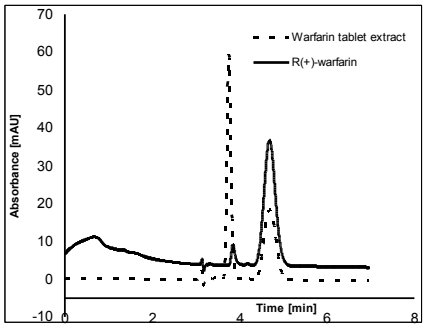

Figure 4. Chiral HPLC separation of warfarin tablet extract (- - -) and standard R(+)-warfarin (—)

Conclusions

The obtained results demonstrate once again the high analytical value of multivariate data analysis in general, and of the regression modeling in particular. Projection to latent structures by means of partial least squares (PLS) combined with appropriate spectral filters and data pre-processing tools has the ability of extracting quantitative information from apparently messy and complicated spectroscopic data, offering an accurate, non-destructive and high throughput solution in every major domain of pharmaceutical analysis including quality control.

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