Classification and Quantitative Analysis of Azithromycin Tablets by Raman Spectroscopy and Chemometrics

Yan Li, Guorong Du, Wensheng Cai, Xueguang Shao
Research Center for Analytical Sciences, College of Chemistry, Nankai University, Tianjin, China
E-mail: xshao@nankai.edu.cn
Received December 23, 2010; revised January 10, 2011; accepted January 21, 2011

Abstract

Raman spectroscopy has been proven a noninvasive technique with high potential in pharmaceutical industry. In this study, micro Raman technique and chemometric tools were used for identification of azithromycin (AZM) tablets by different manufacturers and quantitative analysis of the active pharmaceutical ingredient (API) in the samples. Support vector machine (SVM), Bayes classifier and $K$-nearest neighbour (KNN) were employed for identification, partial least squares (PLS) regression was used for quantitative determination, and interval partial least squares ($i$PLS) and Monte Carlo based uninformative variable elimination (MC-UVE) methods were used to select informative variables for improving the models. The results show that all the samples can be classified into groups by manufacturers with high accuracy, and the correlation coefficient between the predicted API concentrations and reference values is as high as 0.96. Therefore, micro Raman spectroscopy coupled with chemometrics may be a fast and powerful tool for identification and quantitative determination of pharmaceutical tablets.

Keywords: Azithromycin, Raman Spectroscopy, Pharmaceutical Tablets, Variable Selection, Partial Least Squares (PLS)

1. Introduction

Azithromycin (AZM) is a second-generation macrolide antibiotic. Owing to its superior antibacterial activity, excellent pharmacokinetic properties and few gastrointestinal side effects compared to the first-generation macrolide erythromycin, it has been widely used to treat bacterial infections such as respiratory tract infections, sexually transmitted diseases, skin and soft tissue infections [1]. Therefore, many methods have been developed for detection of AZM, although they require time-consuming treatments prior to measurement.

Vibrational spectroscopic techniques, such as infrared (IR), near infrared (NIR) and Raman spectroscopy, associated with multivariate methods have been employed widely in pharmaceutical industry due to their convenience and efficiency [2,3]. Applications of Raman spectroscopy in tablet analysis has extensively reported, such as solid-state evaluation, polymorphic forms analysis, investigation of coating characteristics, quantitative determination [7,8], and particularly in the identification of pharmaceutical drugs [9] and counterfeits [10-12]. Moreover, on-line analysis [13] and process analysis [14] by using Raman spectroscopy were also studied.

Application of chemometric techniques has made a large improvement in the performance of Raman spectroscopy based methods for investigation of tablets. de Veij [15] established an automated approach for detection of counterfeit artesunate tablets by utilizing Raman spectroscopy and principal components analysis (PCA) combined with hierarchical cluster analysis. Support vector machines (SVMs) for classification of tablets of different product families employing Raman technique was studied by Roggo et al. [16]. Zhang et al. [17] applied Raman imaging to compare multivariate analysis methods for classification and quantitative analysis of a
model pharmaceutical tablet. Partial least squares discriminant analysis (PLS-DA) models based on Raman spectra were applied in the counterfeit test by de Peinder et al. [18]. Besides, multivariate calibration methods have been applied to construct models for quantification of the active pharmaceutical ingredients (APIs) in tablets based on Raman spectra, such as partial least squares (PLS) [19-22], principal component regression (PCR) [23] and artificial neural networks (ANN) [24].

In this study, AZM tablets from different manufacturers in China were studied by Raman spectroscopy and chemometric methods for identification and quantification. The classification models based on Bayes classifier [25], K-nearest neighbor (KNN) [26,27], SVM [16,28,29] and PLS-DA [18,30,31], were used to determine the manufacturer and commonly used PLS regression was adopted for quantification of the API. In addition, the wavelength selection was investigated for improving the performance of the models. The practicability was demonstrated to use Raman spectroscopy for classification and quantitative analysis of pharmaceutical tablets.

2. Experimental

2.1. Samples

31 AZM tablets of four different production enterprises were analyzed. The samples from the four manufacturers were referred to as A, B, C and D. Because the chemical composition of the excipients may be different for different manufacturer, and variations may exist in different batches of a product, samples from different batches were selected. Concentrations of API in the tablets determined by the reference method (HPLC) were provided by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), ranging from 50% to 60% (w/w).

2.2. Measurement of Raman Spectra

Raman spectra of the samples were obtained with a BRUKER SENTERRA Micro Raman spectrometer (Bruker Optics Inc, Ettlingen, Germany) equipped with charge coupled device (CCD) based on semiconductor refrigeration technology and 785 nm laser. In the experiments, a sample was placed on the microscope carrier, and located on the facula (50 × 1000 µm) of the laser beam passing through the focusing systems. The spectra were collected at a resolution of 8 cm⁻¹, over the wavenumber range of 3500 - 70 cm⁻¹. Each spectrum is composed of 1716 data points. The integration time was 10 s and 5 co-additions were performed at a laser power of 100 mW on the sample. The OPUS (Bruker Optics Inc, Ettlingen, Germany) software was used for data acquisition. The coating of the film-coated tablets was systematically removed with a scalpel before measurements.

2.3. Data Pretreatment

Figure 1(a) shows typical Raman spectra for the tablets in four different categories. It can be seen that the spectral intensity of the samples are quite different from each other, which may be caused by crush size and dispersion effects. Multiplicative scatter correction (MSC) is, therefore, used to correct the drifting baselines. Figure 1(b) shows the spectra after MSC pretreatment. It is apparent that the baseline was corrected in the pretreated spectra.

2.4. Classification and Quantification

Four classifiers were adopted for identification analysis, i.e., Bayes, KNN, SVM and PLS-DA. All the classifiers have been well studied for classification problems. Bayes classifier is similar to linear discriminant analysis (LDA)
that is a linear method particularly suitable for high dimensional problems. KNN classifier is simply based on the distance of the samples making it a common method for classification problems. In this work, 2NN with Euclidean distance was used. SVM is a nonlinear algorithm by a kernel transformation mapping the samples into a high dimensional hyperplane so that the examples of the separate categories can be divided by a clear gap as wide as possible. PLS-DA is the most commonly used method for identification. Quantitative analysis was performed by PLS regression, because it is the most commonly used multivariate calibration tool.

In the calculations, all the samples were divided into calibration and validation set by a ratio of ca. 3:2 using Kennard-Stone method [32]. Therefore, 21 samples were used as calibration set to construct the classification and quantitative models, and the other 10 samples were used as validation set to evaluate the models. Each set covers all the four categories of samples. The factor number for PLS model was determined by the root mean square error of cross validation ($\text{RMSECV}$), which was obtained by leave-one-out cross validation (LOO-CV). The performance of classification model was evaluated by accuracy and PLS model was evaluated in terms of the correlation coefficient ($R$) between the reference and prediction concentration, the $\text{RMSECV}$ and the root mean square error of prediction ($\text{RMSEP}$) of the validation set.

2.5. Variable Selection Methods

Raman spectra are composed of 1716 variables in this study as described above. However, not all the variables have equivalent effect in the model. Some of them may take important role in the model, but others may be uninformative. Therefore, interval partial least squares (iPLS) [33-35] and Monte Carlo based uninformative variable elimination (MC-UVE) [36,37] were used for variable selection for classification and quantitative modeling, respectively. iPLS subdivides the data into non-overlapping sections that each undergoes a separate PLS modeling, and the useful variable ranges are determined by the value of $\text{RMSECV}$ of the local models. The stability was used in MC-UVE method to evaluate the importance of each variable in the models, so that those variables with larger stability are known as informative and used in the modeling. It is worthy of noting that, in the variable selection for quantitative models, the same way as in the literatures [36,37] was used, for the classification models, however, the concentration vector was replaced by the class number of the samples. The class number for the samples of class A, B, C, and D were assigned as 1, 2, 3 and 4, respectively.

3. Results and Discussions

3.1. Raman Spectra of AZM Tablets

Figure 2 shows a typical Raman spectrum of the samples in category B, on which the vibrational bands assigned to API and excipients are marked respectively. The tentative assignments are based on the comparison with the published data in literatures [38-40]. The regions around 2934, 780 and 734 cm$^{-1}$ are related to the CH vibrations, which are arisen from API and excipients in tablets. The band at 1720 cm$^{-1}$ corresponds to CO double bond vibration, which is mainly caused by AZM in tablets. Meanwhile, the peaks between 1500 and 1000 cm$^{-1}$ should be due to coupled vibrations in AZM. However, the Raman bands at 968, 870 and 404 cm$^{-1}$ may be caused by PO vibration, which is due to phosphate used as diluent in pharmaceutical preparations. The Raman bands at 668 and 356 cm$^{-1}$ can be assigned to SiO vibration in talc, which is commonly used in oral solid dosage formulations as a lubricant and diluent. The band at 616 cm$^{-1}$ may be caused by the TiO$_2$, which is used in pharmaceutical formulations as a whitener [40]. The Raman band around 474 cm$^{-1}$ can be assigned to the CC backbone stretch in starch [40], which is the main excipient in producing pharmaceutical formulations. Furthermore, the bands between 400 and 200 cm$^{-1}$ and the strong band at 110 cm$^{-1}$ are probably caused by a combination of API and excipients in tablets.

3.2. Classification

Table 1 summarized the classification results of the samples in the validation set by Bayes, KNN, SVM and PLS-DA, both the results obtained with full-spectrum
and selected wavenumbers by iPLS (partial-spectrum) were listed. It is clear that, when the full-spectrum was used, the accuracies are less than 80.00% for all the four methods. The results indicate that not all the wavenumbers in the spectra are informative for the classification, wavenumber selection is needed.

For the purpose of improving the accuracy of classification, iPLS was adopted to select informative variables. Figure 3 shows the result obtained by iPLS and the dot line shows the value of the full-spectrum model. It can be seen that, most of local models are inferior to the full-spectrum model and only the models of interval No. 2, 3, 4 and 17 surpass the full-spectrum model. Therefore, the 343 variables in the four intervals (two regions from 242 to 756 cm\(^{-1}\) and from 2822 to 2990 cm\(^{-1}\)) were selected to build the classification model. It is clear that the region from 242 to 756 cm\(^{-1}\) is the information of inorganic substances of the excipients in the samples, and the band of 2822 - 2990 cm\(^{-1}\) involves the vibration of CH, which is related to the API in the samples.

After variable selection, the classification accuracy of PLS-DA reaches 100% as shown in Table 1. The results show that Raman spectroscopy with the aid of chemometrics may be a convenient tool for identification of pharmaceutical tablets. However, the variable selection has no significant improvement for the classification accuracies of the other three methods. The reason may be that the variables selection is done with iPLS, in which the performance of each variable region is evaluated by using the coefficients of the PLS-DA model. New methods should be developed for improving the results of the classifiers.

### 3.3. Quantification

At first, PLS model was built by using the 21 calibration samples and full-spectrum data. The number of latent variables used in this model was determined by leave-one-out cross validation. The predicted results are summarized in the first line of Table 2. It can be seen that the correlation coefficient (\(R\)) between reference contents and prediction values of the validation set is 0.9271, and the recoveries are in the range of 95.87% - 102.65%.

![Figure 3. RMSECVs obtained by iPLS for the 20 local models. The dot line indicates the RMSECV of the full-spectrum model.](image)

### Table 1. Classification results for the samples in the validation set.

<table>
<thead>
<tr>
<th>Classifier</th>
<th>Classification criteria</th>
<th>Full-spectrum</th>
<th>Partial-spectrum ((N))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bayes</td>
<td>Diaglinear(^b)</td>
<td>70.00</td>
<td>70.00 (343)</td>
</tr>
<tr>
<td>KNN</td>
<td>Euclidean distance(^c)</td>
<td>80.00</td>
<td>60.00 (343)</td>
</tr>
<tr>
<td>SVM</td>
<td>Linear kernel(^d)</td>
<td>80.00</td>
<td>60.00 (343)</td>
</tr>
<tr>
<td>PLS-DA</td>
<td></td>
<td>80.00</td>
<td>100.00 (343)</td>
</tr>
</tbody>
</table>

\(^a\)\(N\) is the number of wavenumbers used in the model; \(^b\)Diaglinear is a type of discriminant function that calculates the density of each group with a diagonal covariance matrix; \(^c\)Euclidean distance measures the distance of two points using the Pythagorean formula; \(^d\)Linear kernel produces a linear subspace of a homogeneous system with linear equations.

### Table 2. Results of the PLS models for quantification.

<table>
<thead>
<tr>
<th>Spectral range</th>
<th>Latent variable number</th>
<th>(RMSEP)</th>
<th>(R)</th>
<th>Recoveries (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-spectrum</td>
<td>5</td>
<td>0.0120</td>
<td>0.9271</td>
<td>95.87 - 102.65</td>
</tr>
<tr>
<td>Partial-spectrum</td>
<td>((N = 135))</td>
<td>0.0080</td>
<td>0.9634</td>
<td>98.29 - 102.65</td>
</tr>
</tbody>
</table>
Although the results obtained with full-spectrum data are acceptable, variable selection was performed by using the MC-UVE method for the purpose of simplifying and further improving the PLS model. Figure 4 shows the distribution of the stability of each variable in the wavenumber 70 - 3500 cm\(^{-1}\) by MC-UVE method. Clearly, there are lots of the variables whose stability absolute values are very small. This indicates that most of the variables are uninformative and can be eliminated, and quantification model with those variables whose stability absolute values are comparatively large should be better. Therefore, the variation of the \(RMSECV\) with the number of the retained variables was investigated. Figure 5 shows the \(RMSECV\) obtained with different number of variables selected according to the stability values. It can be seen that, when \(N\) is 135, the lowest value of \(RMSECV\) is obtained. In order to build a parsimonious PLS model, 135 variables are used, and the threshold determined by the number is marked with dot lines in Figure 4. It can be found that the selected variables are concentrated on the bands below 1500 cm\(^{-1}\), four broad bands around 712, 1168, 1206 and 1468 cm\(^{-1}\), some narrow wavelength intervals around 190, 316, 602, 618, 736, 974, 1002 and 1056 cm\(^{-1}\), and several wavenumbers around 2610 and 2768 cm\(^{-1}\). The bands in these regions are mainly related to the API in tablets.

The second line of Table 2 shows the predicted results of the validation set with the simplified PLS model. The correlation coefficient and the recoveries are improved to 0.9634 and 98.29% - 102.65%, respectively. Compared with the full-spectrum model, the results are further improved. Table 3 demonstrates the prediction error for each sample in the validation set. All these values can be found to be within 3% (the maximum is 2.65% corresponding to the maximal recovery of 102.65%). Such results clearly show that Raman spectroscopy with the aid of chemometrics is a convenient and precise tool for quantification of API in tablets.

### 4. Conclusions

The flexibility of identification and quantitative determination of AZM tablets by using micro Raman spectroscopy is investigated. The accuracy of a PLS-DA model with full-spectral data is as high as 80.00%, and the model with the variables selected by \(iPLS\) can reach 100%. For quantitative determination, the full-spectrum PLS model produces prediction accuracy of 95.87% - 102.65%, and the partial-spectrum PLS model with the variables selected by MC-UVE can further improve the prediction accuracy to 98.29% - 102.65%. Therefore, this study demonstrates that Raman spectroscopy coupled with appropriate chemometric methods provides a convenient way for fast identification and quantification of tablets. The method may take an important role for the quality control of release pharmaceutical tablets.

<table>
<thead>
<tr>
<th>Class</th>
<th>Sample No.</th>
<th>Reference (% w/w)</th>
<th>PLS model (% w/w)</th>
<th>Relative err (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>6</td>
<td>57.10</td>
<td>58.61</td>
<td>2.65</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>57.10</td>
<td>57.89</td>
<td>1.38</td>
</tr>
<tr>
<td>D</td>
<td>12</td>
<td>59.82</td>
<td>59.66</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>51.93</td>
<td>51.71</td>
<td>0.43</td>
</tr>
<tr>
<td>A</td>
<td>18</td>
<td>51.93</td>
<td>52.84</td>
<td>1.76</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>51.42</td>
<td>52.02</td>
<td>1.18</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>52.95</td>
<td>53.52</td>
<td>1.07</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>53.72</td>
<td>52.80</td>
<td>1.71</td>
</tr>
<tr>
<td>B</td>
<td>29</td>
<td>53.97</td>
<td>53.13</td>
<td>1.55</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>54.68</td>
<td>55.34</td>
<td>1.21</td>
</tr>
</tbody>
</table>
5. Acknowledgements

The authors thank Dr. Feng, National Institute for the Control of Pharmaceutical and Biological Products, for providing the samples and the related information, and Bruker Optics-Beijing for instrumental support. This study is supported by National Natural Science Foundation of China (No. 20835002).

6. References


