SPECTRAL DATA ANALYSIS FOR A COMPLEX DRUG MIXTURE CONTAINING ALTIZIDE, POTASSIUM CANRENOATE, AND RESCINNAMINE

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Conventional and chemometric spectrophotometric techniques were compared for their analytical performance in determining a tri-component pharmaceutical mixture containing altizide, potassium canrenoate, and rescinnamine. These components were characterized by a notable spectral overlap, thus making their quantitative determination particularly difficult. The determination of altizide and canrenoate was performed using the technique of different order-derivative spectrophotometry, while rescinnamine was determined by fluorometry with activation and fluorescence maxima respectively at 325 and 427 nm thanks to a total absence of interference from the other two components. The analysis of the mixture was carried out by applying multivariate calibration methods, including principal component (PCR) and partial least squares (PLS) regression approaches. The calibration sample set was defined by a simplex-lattice experimental design to cover the experimental domain distributed over five concentration levels. The prediction accuracy of the defined methods was evaluated through external validation on new unknown samples and commercial pharmaceuticals. Significant advantages were found in the prediction of all the analytes when using the chemometric methods, which proved to be simpler, faster, and showing better statistical results with accuracy values between 96.12 and 103.36% and relative standard errors lower than 1.7%.

Keywords: *drug analysis, derivative spectrophotometry, fluorometry, principal component regression, partial least squares regression.*

ОБРАБОТКА СПЕКТРАЛЬНЫХ ДАННЫХ ДЛЯ СЛОЖНОЙ ЛЕКАРСТВЕННОЙ СМЕСИ, СОДЕРЖАЩЕЙ АЛТИЗИД, КАНРЕНОАТ КАЛИЯ И РЕСЦИННАМИН

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Проведено сравнение общепринятых и хемометрических спектрофотометрических методов по их аналитическим характеристикам при анализе трехкомпонентной фармацевтической смеси, содержащей альтизид, канреноат калия и ресциннамин. Для этих компонентов характерно заметное перекрытие спектров, что особенно затрудняет их количественный анализ. Определение содержания альтизида и канреноата проведено методом спектрофотометрии производных разного порядка, ресциннамина – с помощью флуорометрии с максимумами возбуждения и флуоресценции при 325 и 427 нм из-за полного отсутствия помех от двух других компонентов. Смесь проанализирована с применением методов многомерной калибровки, включая регрессию с использованием главных компонент и частичных наименьших квадратов. Калибровочный набор образцов определен экспериментальной схемой с симплексной решеткой, охватывающей область экспериментальных данных, распределенную по пяти уровням концентрации. Точность прогнозирования используемых методов оценена путем внешней валидации на новых неизвестных образцах и коммерческих фармацевтических препаратах. Установлено, что при прогнозировании всех аналитов значительными преимуществами обладают хемометрические методы, которые оказались более простыми, быстрыми и по-

казывающими лучшие статистические результаты с точностью от 96.12 до 103.36% и относительной стандартной ошибкой <1.7%.

Ключевые слова: *анализ лекарственных препаратов, спектрофотометрия производных, флуориметрия, регрессия главных компонент, частичная регрессия наименьших квадратов.*

Introduction. Among the pharmaceutical forms used in antihypertensive therapy, the combination of altizide (ALT), potassium canrenoate (CAN), and rescinnamine (RES) has proved to be successful for the synergy of diuretic and antihypertensive action. Similar to thiazide congeners, ALT has a diuretic effect acting on the proximal part of the distal tubule of the nephron, increasing the excretion of sodium and the volume of urine [1]. CAN is a competitive aldosterone antagonist with an inhibitory effect on aldosterone receptors and other mineralocorticoids in the tubular cells [2]. Along with reserpine from *Rauwolfia serpentina*, RES is the most important alkaloid used as a hypotensive drug, as well as in the treatment of some cases of psychic agitation [3].

The simultaneous determination of spironolactone, a prodrug of CAN and ALT, in a pharmaceutical preparation has been described by applying the partial least squares (PLS) procedure to the data from spectrophotometric analysis [4]. RES has been quantified by colorimetry or fluorometry [5–7]. Other methods for the determination of these drugs are very old; no analytical methods have been proposed for the simultaneous quantitative determination of these compounds in pharmaceutical forms in recent years.

Since UV spectrophotometry has often shown difficulties in the analysis of multicomponent mixtures due to the low resolution power, it has been replaced by chromatographic techniques, especially in the pharmaceutical field [8–10]. However, its use is still widespread thanks to its simplicity both in sample preparation and instrumental use. On the other hand, derivative spectrophotometry was successfully introduced a few years ago to overcome the limitation of UV spectrophotometric techniques owing to its capability of processing spectra composed of overlapping bands, thus also solving multicomponent mixtures [11–14]. Furthermore, its capability of eliminating the basic interference of complex matrices, such as biological ones, has also contributed to making this technique successful [15, 16].

In recent years, the chemometric processing of data has been another major driver of the use of UV spectrophotometry. These methods allow to build mathematical models by simultaneously using data from the full spectra and sample concentrations [17]. The most commonly used multivariate methods in pharmaceutical analysis are principal component regression (PCR) and partial least squares regression (PLS), both described extensively in a number of papers or reviews [18–21]. Mathematical models defined by chemometric techniques are applied on new and unknown samples to predict their composition.

Many multicomponent pharmaceutical formulations with overlapping spectra have been solved thanks to the chemometric processing of spectral data [22, 23]. The models can also be optimized by selecting signals at wavelengths that are richest in analytical information or by eliminating those carrying noise or interference. In some cases, chemometric modelling has been applied to derivative spectrophotometry data, demonstrating great ability in solving multicomponent matrices with severe spectra overlap [24–26].

This paper describes a comparative study of conventional spectrophotometric methods and multivariate procedures based on PCR and PLS algorithms, applied to the absorbance data of ALT-CAN-RES pharmaceutical mixtures. The definition of the calibration set was assisted by design of experiment (DOE) techniques in order to cover the entire experimental domain and then increase the predictive capacity of the calibration models [27, 28]. In the present work, the simplex-lattice design approach was adopted, defining a series of standard mixtures centered on the composition of the pharmaceutical formulation.

The models were validated on a set of prediction samples. Both the methods defined with traditional spectrophotometric techniques and those combined with chemometric processing were applied to analyze the ternary mixture in the commercial pharmaceutical form. Their performance was then compared in order to establish their reliability in terms of precision and accuracy.

Experimental. *Reagents and chemicals.* Ethanol was of spectroscopic reagent grade. ALT, RES, and CAN were of analytical reagent grade (SIGMA Chemical Company, Milan, Italy).

Pharmaceutical formulations. Aldatense® tablets (SPA, Milan, Italy) containing 20.0 mg ALT, 30.0 mg CAN 30.0, 1.00 mg RES 1.00, 30 mg starch, 10 mg talc 10, and 110 mg lactose was analyzed.

Instruments. Spectrophotometric analysis was performed using an Agilent 8453 UV spectrophotometer with a diode array detector (Agilent Technologies, CA, USA) under the following conditions: 10 mm quartz cell; wavelength range 200–400 nm; spectral band 1 nm. Derivative spectra were elaborated by the Savitzky–Golay algorithm with a derivative order 1, polynomial order 2, and a $\Delta\lambda$ value fixed at 4 nm. The soft-

ware UV-Visible ChemStation A.10.01 (Agilent Technologies, CA, USA) was used for the spectral acquisition and elaboration. The application of the chemometric algorithms was supported by the software package "The Unscrambler X 10.3" (Camo Process As., Oslo, Norway). Fluorescence spectra were made at room temperature on a Perkin-Elmer Model MPF-44 B fluorescence spectrophotometer. Excitation wavelength 325 nm (spectral slit width 10 nm); emission wavelength 427 nm (spectral slit width 3 nm). Scan speed 240 nm/min; response time 0.3 s.

Calibration and prediction samples. An accurate building of the calibration set can affect the prediction ability of the regression models. Statistical DOE was adopted to minimize the number of samples and optimize the component concentrations covering the full experimental region. This region was defined through a Simplex-lattice design as a triangular surface [29]. The simplex model distributed the three drugs on five concentration levels, thus building a well-balanced calibration set. The absorbance of each mixture was assured not to exceed the linear range of the spectrophotometer.

Stock solutions of 30 mg/mL ALT, 20 mg/mL CAN, and 1 mg/mL RES in ethanol were used to set up the calibration set samples. The stock solutions were diluted with ethanol to give a set of 17 samples with concentrations ranging between 5.0–30.0 μg/mL (CAN and ALT) and 0.5–3.5 μg/mL for RES. These values were chosen in agreement with the concentration ratio of the commercial formulations. The composition of the solutions is listed in Table 1, while the centroid simplex scheme is shown in Fig. 1. A series of synthetic mixtures containing binary or ternary mixtures of ALT, CAN, and RES was analyzed in order to test the prediction ability of the different analytical strategies. A concentration set design of 10 samples containing the drugs with the same concentration ranges used in the calibration set was prepared.

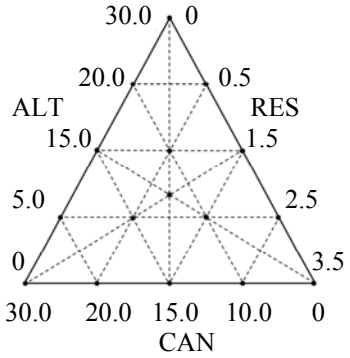

Fig. 1. Centroid simplex scheme for ternary mixture ALT, CAN, and RES.

TABLE 1. Calibration Samples from Simplex-lattice Design with Drug Concentrations (μg/mL) Distributed on Five Levels

Samples	ALT	CAN	RES
1	30.00	0.00	0.00
2	20.00	5.00	0.00
3	20.00	0.00	0.50
4	15.00	15.00	0.00
5	15.00	5.00	0.50
6	15.00	0.00	1.50
7	5.00	20.00	0.00
8	5.00	15.00	0.50
9	5.00	5.00	1.50
10	5.00	0.00	2.50
11	0.00	30.00	0.00
12	0.00	20.00	0.50
13	0.00	15.00	2.50
14	0.00	5.00	2.50
15	0.00	0.00	3.50
16	10.00	10.00	1.00
17	10.00	10.00	1.00

N o t e. The concentration values were selected in accordance with commercial formulations ratio.

Pharmaceutical samples. A sample of five tablets was weighed and reduced to a fine powder. An amount corresponding to one tablet was suspended in ethanol and made up to a volume of 10 mL, sonicated for 10 min, and then filtered through a PTFE 0.45 μm membrane filter. The samples for analysis were obtained after suitable dilution with ethanol to obtain samples with concentration within the ranges of the calibration samples.

Multivariate analysis. PCR and PLS methods were used to build a mathematical model by using the analytical data and the component concentrations from a set of calibration samples. Accordingly, this model is applied to predict the concentrations of other samples. Both PCR and PLS algorithms use linear combinations of the original variables to obtain fewer variables, called principal components (PCs) or factors. The combination of the PCs allows one to build the mathematical model.

The prediction performance of the model is influenced by the number of PCs that has to be optimized. Usually, the first PCs contain most of the information, but it cannot be excluded a priori that the other PCs also contain useful information. Hence, the model needs to be appropriately validated. The full cross-validation procedure was therefore adopted in this work, whereby one reference at a time is removed from the calibration set, and the same sample is subsequently predicted by using the calibration built with the other references. The optimal number of PCs associated with the model's best prediction ability corresponded to the lowest prediction error obtained and to a correlation coefficient (R^2) close to value 1. The prediction error was expressed by the root mean square error of cross validation (RMSECV)

$$\text{RMSECV or RMSEP} = \sqrt{\frac{\sum_{i=1}^{n}(c_i - \hat{c}_i)^2}{n}},$$

where $\hat{c}$ is the predicted concentration; c_i is the real value of concentration; n is the number of calibration samples.

The prediction performance of the multivariate models was evaluated by external validation on new samples (not enclosed in the calibration step). The results obtained were discussed by comparing the figures of merit of the root mean square error of prediction (RMSEP) and percentage recovery (% Recovery).

Results and discussion. Figure 2 shows the absorbance spectra of the ethanol solutions of pure ALT, CAN, and RES with concentration values equivalent to the content of a commercial pharmaceutical formulation. Analysis of the mixture by ordinary spectrophotometry appears difficult to apply due to the large overlap of the peaks. No characteristic signals assigned to individual components can, in fact, be identified in any part of the spectrum. The greatest difficulties were found in the determination of RES, whose relative amount is considerably lower than that of the other two components.

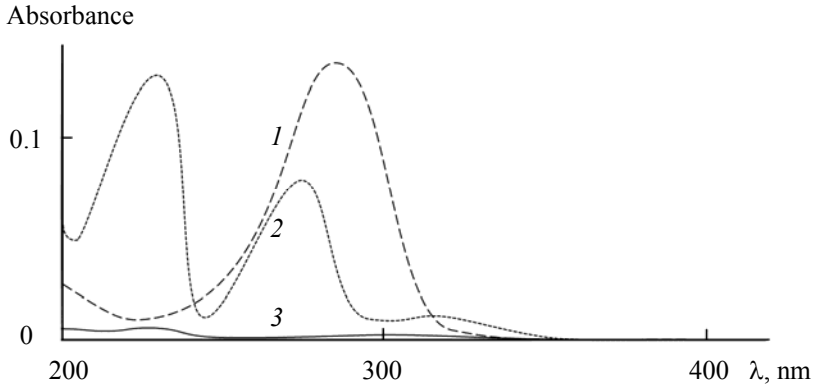

Fig. 2. Absorbance spectra of drugs ALT (1), CAN (2), and RES (3) in ethanol solution.

Analysis by conventional techniques. The derivative spectrophotometry technique was adopted to determine ALT and CAN as the components with the highest concentration in the pharmaceutical mixture. The mathematical derivative values of the absorbance signals against wavelengths ($\Delta A/\Delta\lambda$) were calculated. The wavelength interval ($\Delta\lambda$) to calculate the derivative spectra was varied, and a value of 4 nm was eventually selected since it showed the best signal-to-noise ratio.

In the first-order derivative spectrum, signals attributable exclusively to these two drugs and having no influence in the first-order derivative spectrum, and signals attributable exclusively to the two drugs and

without the influence of the absorbance of the others, were, in fact, identified. The simultaneous determination of ALT and CAN was performed using the regression relationships calculated between concentrations and amplitudes of the derived signals measured on the calibration samples.

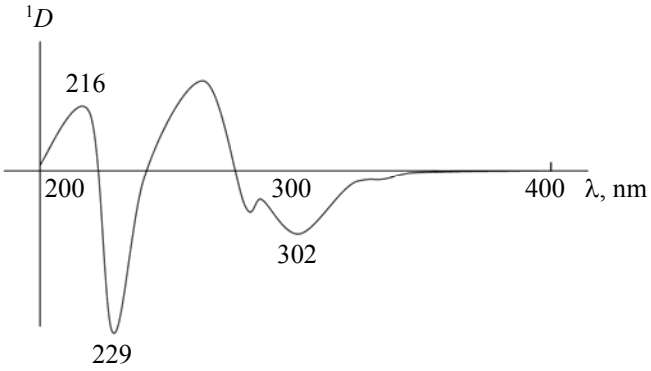

Fig. 3. First-order derivative spectrum of ternary mixture in ethanol solution.

In particular, ALT concentration was perfectly correlated to the $^1D_{216–229}$ signal, and CAN concentration to the $^1D_{302}$ signal, through the following relationships, validated within the range of 5–30 μg/mL:

$$^1D_{216–229} = 10.587\ [\text{ALT}] + 1.325\ (R^2 = 0.998),$$
$$^1D_{302} = 2.1056\ [\text{CAN}] - 0.785\ (R^2 = 0.999).$$

For the RES determination, both ordinary and derivative UV spectrophotometric techniques were unable to quantify the drug in the low-dosed formulation since the spectrum of this substance was completely covered by the absorbance of the other two compounds. Therefore, the quantitative determination of RES was performed by means of a classical fluorometric procedure, showing the maximum of activation at 325 nm and the maximum of fluorescence at 427 nm. The reading of the RES signal was accomplished through the relative calibration curve within 10 min from sample preparation. After this time, in fact, the fluorescence emission was slowly reduced to a 5% decrease after 1 h. The other compounds did not prove to be fluorescent under the experimental conditions adopted.

The analysis of the calibration samples of RES within the range of 0.5–3.5 μg/mL provided the following calibration equation:

$$\text{Emission}_{427} = 0.2124\ [\text{RES}] + 0.0541,\ R^2 = 0.995.$$

The determination of CAN and ALT gave satisfactory results, showing recovery within the range of 99.65–102.74% and relative standard deviation (RSD) below 2.23%. Unfortunately, RES quantitation was not as satisfactory in terms of both recovery (112.63%) and RSD (until to 8.54%), and this was obviously imputable to the relatively low content in the mixtures (Table 2).

Analysis by chemometric techniques. In Fig. 2, a clear overlap between the absorbance curves of the three components is evident along the full spectral region, and no signal due to a single component can be identified. When a mixture presents such a difficult spectral resolution, it is worth considering whether the application of chemometric techniques can overcome these drawbacks. The multivariate methods can simultaneously process a very high number of variables, which correspond to the absorbance values at all recorded wavelengths in the case of spectrophotometry. In this way, it is possible to extract analytical information from the whole spectrum. The first calibration step allows one to establish a relationship between the concentration values of a sample set and their absorbance values. The built mathematical model will be used in the prediction step to estimate the concentrations of new samples.

The prediction ability of the multivariate model can be maximized by an appropriate building of the calibration set [30, 31]. Accordingly, a centroid simplex lattice design, characterized by each component being distributed over five levels of concentration, was adopted in order to define the calibration standard mixtures covering the experimental domain centered on the pharmaceutical specialty content.

The PCR and PLS models were defined by processing both concentrations and spectral data of the calibration samples. The modelling employed the spectral signals of the full ordinary absorption spectrum between 200 and 400 nm. These models were validated through the full cross-method, thereby obtaining the

statistical parameters RMSECV and R^2 (Table 2). The RMSECV values were both satisfactory, and the PC values were between 3 and 7, with the highest values always referring to the determination of RES, which had the lowest relative concentration. The same prediction difficulties for this component were also evident from the observation of the R^2 values.

TABLE 2. Statistical Data Obtained from Conventional Spectrophotometric, PCR, and PLS Analysis

Parameter	ALT	CAN	RES	ALT	CAN	RES
Conventional spectrophotometric techniques[*]						
Derivative UV analysis			*Fluorometry*			
% Recovery	99.65	102.74	112.63	–	–	–
RSD, %	1.53	2.23	8.54	–	–	–
Multivariate techniques						
PCs	3	4	7	3	4	6
Full cross validation						
RMSECV	0.745	0.806	1.849	0.684	0.741	1.724
R^2	0.995	0.992	0.917	0.996	0.998	0.895
External validation for optimized models[*]						
PCs	3	3	5	3	3	5
RMSEP	0.207	0.184	0.420	0.216	0.099	0.259
% Recovery	101.82	100.71	102.34	100.38	99.39	102.65
RSD, %	1.77	1.67	3.25	1.34	1.85	2.61

[*]Average of five replicates ($n = 5$). Results from internal and external validation of models are listed, and comparison between conventional and chemometric analysis is possible.

By comparing the prediction results obtained from the PCR and PLS algorithms, it was observed that the PCR model showed good RMSECV and R^2 values for ALT and CAN, but less satisfactory ones for RES. The lower performance in the RES prediction could always be attributed to the difficulties associated with the low relative concentration of this component. The PLS model, instead, presented slightly higher RMSECV values for ALT and CAN and unsatisfactory ones for RES. It is likely that PLS processing suffered particularly from the absence of linearity between the spectral signals and the component concentrations, given that this was particularly evident when the spectral signals were very low and easily affected by instrumental noise.

Optimization of the PLS models, which is still useful for any multivariate technique, was attempted by selecting spectral regions characterized by a greater absorbance or a minor overlap of the spectra. Several spectral ranges were tested, but those providing the best results were the regions of 200–240 nm, which were more influenced by the signals of ALT and RES, and the regions of 280–330 nm, in which the signals of CAN and RES were prevalent. The selection of the wavelengths has been particularly addressed to those with a higher RES absorptivity in order to emphasize the analytical information of this component and to compensate its low concentration.

Both PCR and PLS models were rebuilt by using the wavelength regions detailed above. A remarkable reduction of the number of PCs was observed and, concurrently, the statistical RMSECV and R^2 parameters showed improvement. These models were subjected to the external validation test and applied on the prediction samples. Satisfactory results were obtained with recovery within the range of 99.39–102.65%, and with RSD, under 3.3%.

Assay of pharmaceuticals. A commercial pharmaceutical formulation was analyzed following the defined spectrophotometric procedures by applying the conventional methods as well as the chemometric techniques. Results are reported in Table 3, where the calculated statistical parameters are listed. Based on the use of the selected wavelengths, the results obtained from the application of the PCR and PLS models showed no significant differences. In particular, assay results of ALT and CAN present in relatively high quantities were in excellent agreement with the content of the labeled drugs. By contrast, the determination of RES present in low concentration showed slightly better results through the use of the PLS model.

TABLE 3. Statistical Results by Applying Derivative Spectrophotometry ($^1D_{nm}$), Fluorometry (FL), PCR, and PLS Methods to the Analysis of Pharmaceuticals

Parameter	$^1D_{nm}$ analysis		FL	PCR			PLS		
	ALT	CAN	RES	ALT	CAN	RES	ALT	CAN	RES
Actual	20.00	30.00	1.00	20.00	30.00	1.00	20.00	30.00	1.00
Mean*	21.74	29.82	4.38	20.72	30.75	5.32	20.03	30.53	5.22
% Recovery	95.36	96.02	92.20	99.68	101.80	103.28	96.12	98.18	103.36
RSD, %	2.07	3.56	5.40	1.28	1.69	1.49	1.55	1.12	1.46

*Expressed as mg per tablet and are referred to average of five replicates (n = 5).

The best performance of PLS can be due to the fact that this algorithm performs analysis for each component, correlating the variation of component information with respect to the overall spectral data. Hence, PLS was able to appreciate even small differences in the spectral data, such as those due to the contribution of a component present in minimum concentration. Thus, the PLS technique can built a stronger model than the PCR one and with a greater predictive power.

The results of analysis of pharmaceutical samples by conventional analysis, derivative spectrophotometry coupled to fluorometry, appeared to be less satisfactory than those obtained through the use of the multivariate techniques. Accuracy values were within the range of 95.36 and 99.68, respectively, for ALT and CAN, whereas for the determination of RES they showed an average recovery value of 92%. Even in terms of precision, the results were poor, with RSD values up to 5.4%.

Conclusions. When applied to spectrophotometric data, chemometric methods such as multivariate PCR and PLS techniques have proved effective in the resolution of a complex mixture characterized by a strong overlap of spectral curves, and a particular powerful analytical tool in low-dosed drugs. Compared to traditional spectrophotometric methods, the main advantages are simplicity and speed, as well as the unnecessary presence of sophisticated tools or preventive separation procedures.

In the current work, the results were satisfactory and more reliable and reproducible when compared to the application of classical methods. Nevertheless, chemometric methods require the use of appropriate software for mathematical calculations. In addition, accurate model optimization is necessary in order to achieve the best resolution performance. The construction phase of a multivariate model is first and foremost fundamental as it demands a careful selection of the calibration samples. Consequently, it is preferable to use an appropriate experimental design. The prediction ability of the models can be optimized through an appropriate selection of the number of factors and of wavelengths that are rich in analytical information and free from irrelevant or noisy signals.

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