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A spectrophotometric-partial least squares (PLS-1) method for the simultaneous determination of furosemide and amiloride hydrochloride in pharmaceutical formulations

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Abstract

A numerical method, based on the use of spectrophotometric data coupled to PLS-1 multivariate calibration, is reported for the simultaneous determination of furosemide and amiloride hydrochloride in synthetic samples and commercial tablets. The method was applied in the concentration ranges of 8.0–13.0 mg l^{-1} for furosemide and 1.0–1.6 mg l^{-1} for amiloride hydrochloride. Its accuracy and precision were determined, and it was validated by the analysis of synthetic mixtures of both drugs. The method was successfully applied to the quantitation of furosemide and amiloride hydrochloride in three different pharmaceutical formulations, providing results in agreement with those obtained by HPLC. It allowed the rapid, accurate and precise simultaneous estimation of the concentration of both analytes of interest in spite of their important spectral overlap, high concentration relationship and the presence of small amounts of different, unmodelled, absorbing excipients.

Keywords: Amiloride hydrochloride; Furosemide; PLS-1; Simultaneous determination; Spectrophotometric method

1. Introduction

Since UV-visible spectrophotometry is a rapid, sensitive and inexpensive analytical tool, it is appropriate for dosage control of pharmaceutical preparations. However, the lack of specificity of the UV-visible absorption usually hinders the application of this technique in case of mixtures of absorbing species, due to spectral overlap.

Numerical methods based on the mathematical resolution of multivariate signals, such as UV-visible spectroscopic data, have been shown to allow the resolution of complex mixtures with high speed and acceptable accuracy and precision. Among them, the partial least-squares regression with a single dependent variable (PLS-1) has found important applications in pharmaceutical analysis [1], providing an interesting alternative to the more demanding chromatographic techniques.

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Like other multivariate regression methods, PLS-1 enables the development of mathematical models that relate multivariate instrumental responses such as spectral intensities (r_j) from many calibration samples to their known analyte concentrations (c_i), according to Eq. (1), where e_i is the residual associated with the concentration of the ith sample.

$$c_i = f(r_1, r_2, \ldots, r_j) + e_i. \quad (1)$$

Application of this method comprises two separate stages. The first step is calibration, where the relationship of Eq. (1) is obtained, by spectral evaluation of samples of known analyte concentration, while during the second stage (prediction), this relationship is used to predict analyte concentrations in unknown samples from their instrumental responses.

PLS-1 is an indirect calibration method that does not require individual spectra of each analyte and interference to be known in advance. Hence, it has been successfully applied in pharmaceutical analysis of multicomponent drug products [1–5].

Furosemide (frusemide, 4-chloro-*N*-furfuryl-5-sulphamoylanthranilic acid, FU) is a white or slightly yellow powder, practically insoluble in water but sparingly soluble in methyl alcohol (MeOH) and soluble in aqueous alkaline solutions. This drug is a potent diuretic that inhibits the reabsorption of electrolytes in the ascending limb of the loop of Henle and also in the renal tubules. While FU has no clinically significant effect on carbonic anhydrase, it enhances water excretion, increasing loss of sodium, chloride and potassium ions [6].

However, amiloride hydrochloride (*N*-amidino-3,5-diamino-6-chloropyrazine-2-carboxamide hydrochloride, AM), available as the dihydrate, is a light-sensitive yellow powder, sparingly soluble in MeOH and slightly soluble in water, giving slightly acidic solutions. It is a mild diuretic that appears to act mainly on the distal renal tubules; like spironolactone, it increases the excretion of sodium and chloride ions, while sparing potassium [6].

The association of FU and AM (Fig. 1) furnishes a valuable natriuretic agent with a diminished kaliuretic effect, minimizing the risk of alkalosis in the treatment of refractory oedema associated with hepatic cirrhosis or congestive hearth failure [6]. Both individual drugs are official in the USP 24, Ph. Eur. 3rd Ed. and the BP 98.

Being widely used drugs, several methods have been reported for their determination in pharmaceutical formulations. In the case of FU, these include spectrophotometry [7], fluorescence spectroscopy [8], coulometry [9] and HPLC [10].

Likewise, AM has been determined in pharmaceutical preparations by spectrophotometry [11], TLC-densitometry [12], atomic absorption spectrometry [13], spectrofluorometry [14], differential pulse polarography [15] and HPLC [16].

Methods for the simultaneous determination of both drugs in pharmaceutical dosage forms, however, are scarce and most of them time-consuming; the few reported strategies involve colorimetric [17,18] and HPLC [19–21] techniques. Interestingly, a handful of HPLC systems have been described for the simultaneous evaluation of FU and AM in urine and other biological fluids [22–27], but multivariate calibration strategies have not been applied for the analysis of mixtures of these two drugs.

Described in this paper is a rapid, precise and accurate procedure for the simultaneous determi-

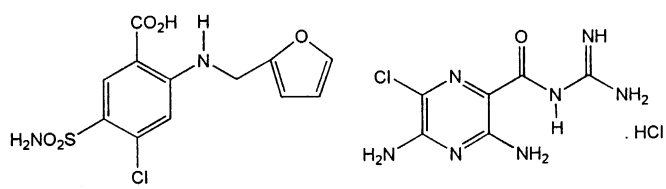

Fig. 1. Chemical structures of furosemide and amiloride hydrochloride.

nation of furosemide and amiloride hydrochloride in synthetic samples and commercial combined tablet preparations, based on the joint use of the PLS-1 algorithm and UV-visible spectrophotometric data.

2. Experimental

2.1. Materials

All experiments were performed with pharmaceutical-grade FU and AM (as the dihydrate) and analytical-grade reagents. Tablets containing FU and AM were kindly supplied by their respective manufacturers. All the preparations (120–180 mg per tablet for the different brands) contain 40 mg of FU, 5 mg of AM and excipients (Aerosil 200, magnesium stearate, lactose, talk, microcrystalline cellulose, and corn starch), including small amounts of different colouring agents, such as quinoline yellow (CI 47005) and FD&C red No. 40. MeOH was generously provided by Resinfor Metanol (Puerto Gral. San Martín, Argentina) and used as received. Stock solutions of FU (504 mg l^{-1}) and AM (303 mg l^{-1}) were prepared by dissolving accurately weighed amounts of the drugs in MeOH–H_2O (1:1, v/v). HPLC-grade reagents were employed for HPLC analyses.

2.2. Methods

2.2.1. Calibration system

A four-level full factorial design training set of 16 samples was prepared for calibration, by convenient dilution of the stock solutions of FU and AM in MeOH–H_2O (1:1) to final concentrations in the range of 8.0–13.0 mg l^{-1} for FU and 1.0–1.6 mg l^{-1} for AM. The analyte levels were chosen to cover the range of $100 \pm 30\%$ of the expected amount of analytes in the unknowns [28] and at such concentrations that they lie in their known linear absorbance-concentration zones.

2.2.2. Validation sets

A validation set of 12 synthetic samples was prepared. Groups of four samples were analysed in three consecutive weeks, allowing the evaluation of inter- and intra-assay precision. A second set of 16 synthetic samples, covering the whole concentration range of interest of both analytes, was prepared for evaluation of accuracy over the linear range of interest.

2.2.3. Sample preparation

Commercial pharmaceutical formulations of three different brands were evaluated. In each case, groups of five tablets were individually weighed, finely powdered and mixed. Portions of the powder (40–60 mg) equivalent to about 13.1 mg of FU and 1.6 mg of AM were accurately weighed and transferred to 50 ml volumetric flasks using 25 ml of MeOH. After being continuously shaken for 30 min, the flasks were made up to volume with distilled H_2O, and the solids were left to decant for 30 min; then, 1 ml aliquots were transferred from each flask to 25 ml volumetric flasks and completed to volume with MeOH–H_2O (1:1). Binary synthetic mixtures of FU and AM were prepared by diluting known amounts of their stock solutions in MeOH–H_2O 1:1, to obtain final concentrations of 10.5 mg l^{-1} FU and 1.3 mg l^{-1} AM.

2.3. Apparatus, hardware and software

Spectrophotometric measurements were carried out with a Beckman DU-640 spectrophotometer, employing a 10 mm quartz cell. Spectra were acquired over the wavelength range 250–350 nm at intervals of 1 nm (101 data points per spectrum) against a blank of solvent, saved in ASCII format and transferred to a PC Pentium II 466 MHz computer for their subsequent manipulation. Samples were analysed in duplicate. PLS-1 data evaluation was performed with an in-house program written for Matlab 5.3 (Mathworks, Inc.), according to Refs. [29,30]. The software was validated against the PLS program MULTIVAR [31], results being in full agreement. The optimum wavelength range was selected by an ad hoc Matlab routine employing a minimum PRESS search through a variable-size moving window [32].

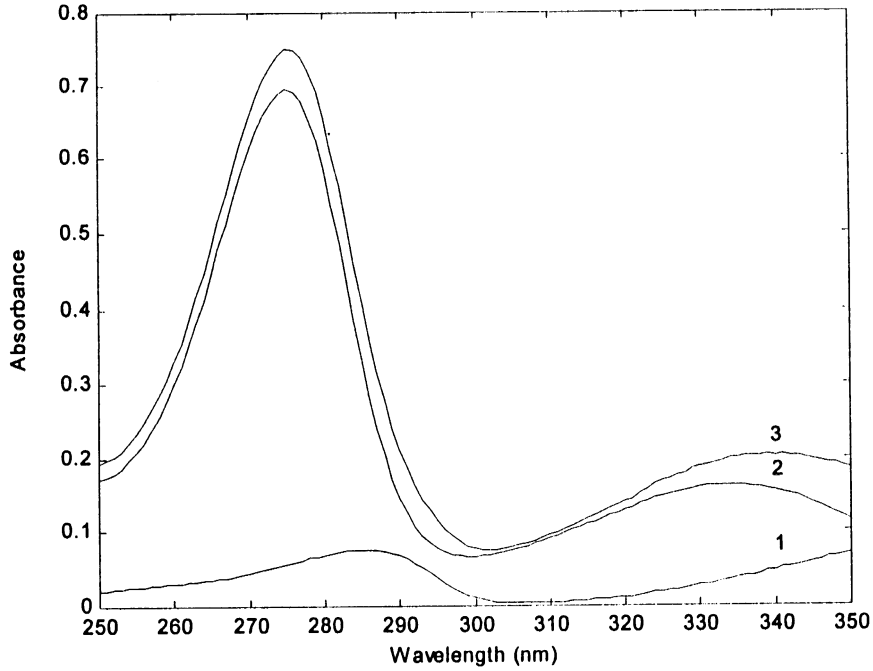

Fig. 2. UV-visible spectra of (1) 1.34 mg l^{-1} amiloride hydrochloride, (2) 10.7 mg l^{-1} furosemide and (3) mixture of furosemide (10.7 mg l^{-1}) and amiloride hydrochloride (1.34 mg l^{-1}).

HPLC analyses of FU and AM were carried out isocratically at ambient temperature with a Gilson liquid chromatograph, equipped with a 307 type pump and a 4.6 mm × 25 cm Spherisorb C-18 column with 5 μm particles. The injection volume was 20 μl, the flow rate was set to 1 ml min^{-1}, and the detection was at 280 nm in both cases, employing a Gilson 112 type UV-visible fixed-wavelength detector. Mobile phases used were MeOH–H_2O–glacial acetic acid (600:388:12, v/v/v) for FU [33] and MeOH–H_2O–phosphate buffer (pH 3; 0.1 M) (568:400:32, v/v/v) for AM [34]. All solvents were filtered through a 0.45 μm Millipore filter and degassed before use. Data were acquired at sensitivity = 0.05 AUFS and a time constant of 0.5 s, and processed on a PC AT 486 DX2 66 MHz computer, employing Gilson's software. Calibration graphs for quantitative determinations were obtained with five different concentrations of authentic samples of FU and AM. Triplicate injections of 20 μl were made for each solution of the unknowns. Concentrations of the unknowns were obtained by interpolation in the calibration graphs.

3. Results and discussion

The electronic absorption spectra of pure FU (10.7 mg l^{-1}) and AM (1.31 mg l^{-1}), as well as the spectrum of a 8:1 mixture (by weight) of both drugs in MeOH-H_2O (1:1) over the wavelength range 250–350 nm are shown in Fig. 2. FU displays an absorption maximum at 274 nm, while AM exhibits a maximum at 285 nm. Data analysis anticipated that the severe spectral overlapping observed coupled to the high FU/AM ratio present in the pharmaceutical preparations could seriously hinder the resolution of the mixture by conventional spectrophotometry.

Erram and Tipnis [21], being aware of this problem, proposed the simultaneous determination of FU and AM by evaluating the absorbance readings at six different wavelengths. However, this proved to be impractical in our case, mainly due to the presence of small amounts of declared absorbing excipients, which were different in the various commercial brands subjected to analysis. In addition, it also precluded the use of derivative

spectrophotometry. Thus, PLS-1 was sought as an alternative, since this method can handle severe spectral overlap and a high analyte concentration ratio, and knowledge of the spectra of all the absorbing species is not absolutely necessary.

The principles of PLS-1 regression have been described in detail in several papers and monographs [29–31]. For a system with m calibration samples, the calibration process with the PLS-1 algorithm involves the simultaneous resolution of matrices $\mathbf{A}$ ($m \times k$) and $\mathbf{C}$ ($m \times 1$). Matrix $\mathbf{A}$ consists of the absorbances (spectra) of the m samples recorded at k wavelengths (sensors), while $\mathbf{C}$ is a vector containing the concentrations of the analyte of interest in the m samples.

$$\mathbf{A} = \mathbf{TP}^T + \mathbf{E} = \sum_{h=1}^{a} \mathbf{t}_h \mathbf{p}_h^T + \mathbf{E} \quad (2)$$

$$\mathbf{C} = \mathbf{UQ}^T + \mathbf{F} = \sum_{h=1}^{a} \mathbf{u}_h \mathbf{q}_h^T + \mathbf{F}. \quad (3)$$

Each of these matrices is resolved into the product of two smaller matrices, which capture the relevant information of the original data, as shown in Eqs. (2) and (3). Matrices $\mathbf{T}$ and $\mathbf{U}$ are the scores matrices of blocks $\mathbf{A}$ and $\mathbf{C}$, respectively; they have dimensions $m \times a$, where a ($a \leq k$) is the number of PLS factors required for proper calibration. Matrices $\mathbf{P}^{\mathrm{T}}$ ($a \times k$) and $\mathbf{Q}^{\mathrm{T}}$ ($a \times 1$), are the loading matrices of $\mathbf{A}$ and $\mathbf{C}$, while $\mathbf{E}$ ($m \times k$) and $\mathbf{F}$ ($m \times 1$) are the residual matrices, which contain the random noise and model error, respectively, when a PLS factors are employed.

During calibration, Eqs. (2) and (3) are solved by least squares. The two resolved matrices are not independent, and the inner relationship $\mathbf{U} = \mathbf{TB}$ is established between the scores of blocks $\mathbf{A}$ and $\mathbf{C}$, where $\mathbf{B}$ is an $a \times a$ matrix. Thus, the loadings for spectral matrix $\mathbf{A}$ contain information from the scores of the concentration matrix $\mathbf{C}$, whereas the scores of the spectral data matrix $\mathbf{A}$ are employed for the calculation of the loadings of the concentration vector $\mathbf{C}$.

Once the scores and loading matrices for both blocks $\mathbf{A}$ and $\mathbf{C}$ have been calculated, prediction of unknowns can be carried out. The component score is obtained from the unknown spectrum $\mathbf{s}_{\mathrm{un}}$ employing Eq. (4); then, the unknown concentration $\mathbf{c}_{\mathrm{un}}$ is calculated using Eq. (5).

$$\mathbf{t} = \mathbf{s}_{\mathrm{un}}\mathbf{P}^T \quad (4)$$

$$\mathbf{c}_{\mathrm{un}} = \mathbf{tBQ}^T. \quad (5)$$

Selection of the optimum number of PLS factors is required to avoid overfitting. This is usually done by cross validation employing the 'leave-one-out' procedure, which involves systematically removing one calibration sample at a time and employing the remaining samples for model building. The concentration of the sample left out is then predicted with the model, and the predicted concentrations of all samples are compared with their actual values. This allows the computation of PRESS, the *prediction error sum of squares* [$\mathrm{PRESS} = \Sigma(C_{\mathrm{act}} - C_{\mathrm{pred}})^2$] for each value of h; the minimum PRESS [PRESS(h*)] is selected and employed in the calculation of the F ratio shown in Eq. (6). According to Haaland and Thomas' proposal [29], the optimum number of factors ($h < h$*) is that corresponding to a probability of less than 75%.

$$F(h) = \mathrm{PRESS}(h)/\mathrm{PRESS}(h^*). \quad (6)$$

Data (calibration set and unknowns) can be pre-processed in order to simplify calculations. Mean centring of the data, a process in which the average calibration spectrum is subtracted from each spectrum and the average calibration concentration is subtracted from each concentration, is frequently employed. This operation often decreases the complexity of the model (less PLS factors required), by eliminating the need to fit a non-zero intercept.

The electronic absorption spectra of the 16 calibration samples of FU and AM were recorded in duplicate in the range 250–350 nm and subjected to PLS-1 modeling. Mean-centred data were used, and the models were cross-validated employing the 'leave-one-out' procedure. Both analytes showed some absorbance at wavelengths greater than 350 nm, but these wavelengths proved not to be necessary to optimal PLS-1 model construction (Table 1).

Although PLS-1 is a full-spectrum technique [29], not all wavelengths carry the same quality of

information; therefore, a variable-size moving window [32] across the spectra was employed for selection of the appropriate regions of interest. For each starting wavelength, models with a variable number of sensors and one to four factors were constructed, and the PRESS was computed in each case. The spectral region affording the minimum PRESS was considered as having the best predictive ability.

Individual components were independently modeled by PLS-1, employing the optimum range of sensors and the optimum number of PLS factors. The selected spectral zones, each including λ_{max} of the respective analytes, demonstrated better results than those obtained using the full absorbing ranges of the calibration samples. The optimum number of factors was chosen for each analyte by application of the *F*-ratio criterion proposed by Haaland and Thomas [29]. Calibration data were also checked for spectral and leverage-related outliers following the criteria of Ref. [30], and a linear response was observed over the examined concentration ranges of both analytes.

Table 1
PLS-1 analysis of FU and AM mixtures: statistical parameters for the calibration

Parameter of interest[a]	Furosemide	Amiloride hydrochloride
Spectral range (nm)	272–301	282–345
Concentration range (mg l^{-1})	8.0–13.0	1.0–1.6
Number of factors	2	3
PRESS (mg l^{-1})^2	0.047	0.0042
RMSD (mg l^{-1})	0.036	0.010
REP (%)	0.34	0.73
r^2	0.9996	0.9983
Selectivity [35]	0.60	0.46
Sensitivity (SEN)	0.118	0.106
Analytical sensitivity [(γ), 1 mg^{-1}]	16.2	45.8
Minimum concentration difference [(γ^{-1}), mg l^{-1}]	0.062	0.022

[a] $\text{PRESS} = \sum_1^I (C_{act} - C_{pred})^2$, $\text{RMSD} = \left[\frac{1}{I}\sum_1^I (C_{act} - C_{pred})^2\right]^{1/2}$,

$\text{REP}\% = \frac{100}{\bar{C}}\left[\frac{1}{I}\sum_1^I (C_{act} - C_{pred})^2\right]^{1/2}$, $r^2 = 1 - \frac{\sum_1^I (C_{act} - C_{pred})^2}{\sum_1^I (C_{act} - \bar{C})^2}$

where $\bar{C}$ is the average component concentration in the I calibration mixtures, Sensitivity $= 1/\|b_k\|$, where b_k is the final regression coefficient vector for component k, and $\gamma =$ (SEN/σ_o), where σ_o is the standard deviation of the blank.

Table 1 summarizes the most relevant statistical parameters of both FU and AM calibration models, including their figures of merit. Critical values of the calibration, such as the root mean square difference (RMSD), the square of the correlation coefficient (r^2) and the relative error of prediction (REP) demonstrated the quality of fit of all the calibration data. It is noteworthy that while two factors were needed in the case of FU, accounting for 99.96% of the spectral variance, the use of three factors, capturing 99.82% of the variance, was required for the correct prediction of AM concentrations, within its optimum spectral range.

The accuracy of the model over the working linear range was assessed, employing an independent validation set of 16 samples, by comparing the amounts obtained in the determination of synthetic samples with those actually added to the solutions. The results of plotting recovered analyte versus added amounts of analyte, summarized in Table 2 indicated that quantitative recovery of the analytes is possible over the range of concentrations tested, satisfying the acceptance criteria for this study. However, precision was determined by means of a one-way ANOVA of a second set of 12 samples, analysed four at a time in three successive weeks.

In comparison with furosemide data, a greater variation of amiloride hydrochloride values was detected, particularly between days. Nevertheless, the results obtained and shown in Table 2 demonstrate that no statistical difference was observed between the means of the different determinations. The mean sample recoveries of this second set were also close to 100%.

The validated PLS-1 calibration model was applied to the simultaneous analysis of FU and AM in synthetic samples and three different commercial tablet formulations from Argentine manufacturers, namely Nuriban-A®, Errolon® and Lasiride®. These drug associations are given to patients suffering from oedema due to congestive

Table 2
Accuracy and precision for the spectrophotometric-PLS-1 simultaneous determination of furosemide and amiloride hydrochloride

Parameter	Furosemide	Amiloride hydrochloride
Accuracy		
N	16	16
Concentration range (mg l^{-1})	8.0–13.0	1.0–1.6
$y_0 \pm$ S.D.	0.0002 ± 0.011	-0.0021 ± 0.0027
Slope $\pm$ S.D.	0.998 ± 0.010	1.02 ± 0.020
R	0.9992	0.9973
Precision[a]		
Between-days variation	4.3×10^{-3}	4.7×10^{-2}
Within-days variation	3.0×10^{-3}	1.25×10^{-2}
F-ratio	1.43	3.76
Mean recovery (%)	99.59	101.04
Between-days RSD (%)	0.57	1.37
Within-days RSD (%)	0.50	1.01

[a] The between-days and within-days degrees of freedom are 2 and 9, respectively. The critical F ratio value for 2 and 9 degrees of freedom is 4.26, at a confidence level of 95%.

hearth failure or either liver or kidney diseases. The prediction results are collected in Tables 3 and 4. As can be seen, the amount of FU was below its nominal content in one of the formulations, and the amount of AM was slightly above its nominal content in two of the commercial brands analysed. Nevertheless, all the values were within their respective specifications and consistent with the manufacturers' labeled contents.

Furthermore, in spite of the relatively unfavourably low concentration of AM in the samples and the low sample absorption in the range 282–345 nm, optimum for the determination of this analyte, this spectrophotometric-PLS-1 method was able to allow its quantitation; highly reproducible recoveries of this drug were obtained in all cases. In order to test further the proposed method, the commercial preparations were evaluated by HPLC, employing slight modifications of published procedures [33,34]. As shown in Tables 3 and 4, mean recovery results obtained employing the proposed spectroscopic method with multivariate calibration were in good agreement with those furnished by HPLC. Statistical comparison of mean recoveries demonstrated no significant differences between both methods, being the spectrophotometric-PLS-1 strategy more simple, con-

Table 3
Spectrophotometric-PLS-1 determination of furosemide in synthetic samples and pharmaceutical preparations: comparison with HPLC

Method	Parameter	Synthetic	Brand 1	Brand 2	Brand 3
	Mean recovery (%)[a]	100.99	92.29	98.52	99.37
UV/PLS-1	RSD (%)	0.49	1.26	1.47	1.00
	SEP (mg l^{-1})[b]	0.05	0.13	0.16	0.11
HPLC[c]	Mean recovery (%)		91.99	97.70	100.44
	RSD (%)		0.65	0.27	0.52
	$t_{(calc)}$[d]		0.401	0.948	1.801

[a] Mean recovery and relative standard deviation relative to nominal content for 24 determinations.

[b] Label claims are 40 mg of furosemide per tablet. The final sample concentrations were approximately 10.5 mg l^{-1}; C_{exp} denotes the expected sample concentrations based on label claims, and C_{pred} denotes their predicted concentrations.

$$\mathrm{SEP} = \left[\frac{1}{N-1}\Sigma_1^N(C_{exp}-C_{pred})-(\overline{C_{exp}-C_{pred}})^2\right]^{1/2}$$ [30,36].

[c] Calibration curve with five points. Regression equation: $c = -0.75 + 129.8 \times 10^{-6}$ AUC; $r = 0.9995$ in the range 5.0–60.2 mg l^{-1}; sample concentrations were approximately 31.5 mg l^{-1}. Mean recovery and RSD of three determinations.

[d] $t_{(25,0.05)} = 2.060$.

Table 4
Spectrophotometric-PLS-1 determination of amiloride hydrochloride in synthetic samples and pharmaceutical preparations: comparison with HPLC

Method	Parameter	Synthetic	Brand 1	Brand 2	Brand 3
UV/PLS-1	Mean recovery (%)[a]	100.06	99.09	105.44	104.29
	RSD (%)	1.79	1.96	2.33	0.83
	SEP (mg l^{-1})[b]	0.023	0.027	0.034	0.028
HPLC[c]	Mean recovery (%)		98.03	104.43	104.65
	RSD (%)[a]		0.33	0.77	0.53
	$t_{(calc)}$[d]		0.929	0.693	0.690

[a] Mean recovery and relative standard deviation relative to nominal content for 24 determinations.
[b] Label claims are 5 mg of amiloride hydrochloride per tablet. The final sample concentrations were approximately 1.3 mg l^{-1}; C_{exp} denotes the expected sample concentrations based on label claims, and C_{pred} denotes their predicted concentrations. $SEP = \left[\frac{1}{N-1}\Sigma_1^N(C_{exp}-C_{pred})-(\overline{C_{exp}-C_{pred}})^2\right]^{1/2}$ [30,36].
[c] Calibration curve with five points. Regression equation: $c = 1.11 + 56.7 \times 10^{-6}$ AUC; $r = 0.9997$ in the range 17.9–89.5 mg l^{-1}; sample concentrations were approximately 52 mg l^{-1}. Mean recovery and RSD of three determinations.
[d] $t_{(25,\ 0.05)} = 2.060$.

venient and less time-consuming, especially in the case of amiloride hydrochloride, where chromatograms required 30 min to develop.

4. Conclusions

A convenient method, based on the use of electronic absorption measurements in conjunction with PLS-1 multivariate calibration analysis, was developed for the simultaneous determination of FU and AM in tablet dosage forms and synthetic binary mixtures. This technique, amenable for routine quality control, requires simple instrumentation, offers high sample throughput and avoids time-consuming separations or complex sample treatments, while providing a good accuracy and precision regardless of the presence of small amounts of different absorbing excipients. The results obtained from the analyses of three pharmaceutical preparations were consistent with those furnished by HPLC runs.

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