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PCA-CR analysis of dissolution profiles. A chemometric approach to probe the polymorphic form of the active pharmaceutical ingredient in a drug product

Rubén M. Maggio, Patricia M. Castellano, Teodoro S. Kaufman*

Pharmaceutical Analysis, Department of Organic Chemistry, School of Pharmaceutical and Biochemical Sciences, National University of Rosario and Institute of Chemistry of Rosario (IQUIR, CONICET-UNR), Suipacha 531, Rosario (S2002LRK), Argentina

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ABSTRACT

A simple chemometric approach to differentiate among the three crystalline polymorphs of the model drug Furosemide (FUR) in a pharmaceutical dosage form is presented. The proposed method is based on the principal component analysis with confidence regions (PCA-CR) comparison of the dissolution profiles of the test pharmaceutical formulation, and formulations containing the different polymorphs, employed as the corresponding references.

For the elaboration of the references, FUR polymorphs I, II and III were prepared, characterized and compounded with the excipients found in the test commercial formulation. The dissolutions were carried out in a discriminating HCl–KCl dissolution medium (pH 2.2), and the corresponding profiles were constructed from the absorbances (274 nm) of the dissolution samples.

PCA-CR was able to differentiate among the three crystalline polymorphs of FUR and to confirm the presence of polymorph I in the test sample, with 99% statistical confidence. The PCA-CR results were compared with those obtained by a bootstrap-mediated implementation of Moore and Flanner's difference factor (f_2). The same conclusion was reached employing an f_2 -based comparison, despite its inability to differentiate between polymorphs II and III. Therefore, PCA-CR may be considered a complementary and useful tool for probing the polymorphic form present in a pharmaceutical formulation.

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1. Introduction

Despite that obtaining drug quality information from multi-component systems such as pharmaceutical formulations is not a straightforward task, there is an increasing demand for new approaches towards this goal, as this may open new perspectives for improvements in the quality control system. The polymorphic form of an active pharmaceutical ingredient is a major concern for pharmaceutical companies because of its influence on physical and chemical properties of drug powders. By affecting drug solubility, dissolution rate and oral absorption, crystal polymorphism may also have impact on product performance, the usability of the final dosage form, and its overall therapeutic efficiency. Therefore, the existence of polymorphic forms in new drug entities must be thoroughly investigated from the early stages of development (Brittain, 1999; Yu et al., 2003) and controlled during manufacture. The most relevant analytical strategies for studying polymorphism include spectroscopic and thermal methodologies, as well as those based on physical and structural properties of solids (Aaltonen et al., 2009).

Furosemide (FUR, Fig. 1) is an efficient diuretic which is widely used for the treatment of hypertension and edema in patients suffering from renal insufficiency, cardiac congestive failure or hepatic cirrhosis (Brunton, 2001).

The drug is a weak acid ($pK_a = 3.8$) of low water solubility, which has three crystalline polymorphs (I, II and III) an amorphous form, two solvates (from dimethylformamide and dioxane, respectively) and an additional crystalline form (VI), stable only at high temperature; none of the latter forms are of use in human medicine (Matsuda and Tatsumi, 1990). The crystal polymorphs of FUR exhibit different chemical stability (De Villiers et al., 1992) and their solid-state ^{13}C NMR spectra exhibited evidence of differences in molecular mobility and structural disorder (Doherty and York, 1989). The drug has poor bioavailability with large variations among and within subjects (Benet, 1979; McNamara et al., 1987) and is characterized by a narrow therapeutic index; therefore the doses of FUR must be carefully regulated in order to avoid risks of electrolytes and water depletion. Because of its low solubility, FUR polymorphism further affects the quality of its drug products, influencing drug absorption and bioavailability (Matsuda and Tatsumi, 1990).

The *in vitro* dissolution test of a solid dosage form is critical for assessing quality and uniformity of the product at the formulation stage and throughout its shelf-life, and useful for quality

* Corresponding author. Tel.: +54 341 4804592x241; fax: +54 341 4370477.
E-mail address: kaufman@iquir-conicet.gov.ar (T.S. Kaufman).

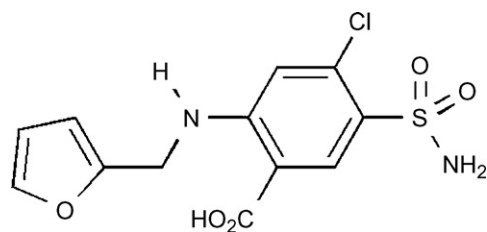


Fig. 1. Chemical structure of Furosemide.

control purposes (Qureshi and McGilveray, 1999). Factors affecting the dissolution rate from dosage forms are classified in four main categories, including those related to the properties of the drug and to the dosage form, as well as factors associated with the dissolution apparatus and test parameters.

Bauer et al. (2002) found that tablets prepared employing FUR from various sources exhibited different dissolution rates. The authors attributed these variations mainly to the polymorphic forms of the drug; differences in particle size and morphology also contributed to the recorded behaviour.

Because of the inherent complexity of the dissolution process, it is often challenging to pinpoint the key factors that mostly affect this process in the final dosage form. Chemometric methods are capable of compressing complex information into a few variables, making it easy to understand the observed phenomena. Strategies based on principal component analysis (PCA) have been suggested for the evaluation of dissolution profiles (Tsong et al., 1997; Adams et al., 2001, 2002) and recently the application of PCA with confidence regions (PCA-CR) as a new approach for the comparison of dissolution profiles and the evaluation of profile similarity between pre-change and post-change batch has been reported (Maggio et al., 2008). Moreover, chemometric methods have started to find widespread use in the analysis of polymorphic forms (Jørgensen et al., 2006; Kogermann et al., 2007, 2008).

Standardization of the dissolution conditions and factors relating to the dosage form and dissolution apparatus converts the dissolution test into a tool capable of correlating the performance of a solid dosage form to the physicochemical properties of its active principle (Swanepoel et al., 2000). Herein, we describe a simple approach to determine the crystalline form present in a pharmaceutical formulation from its dissolution profile, based on the use of PCA-CR as a multivariate chemometric technique, and FUR as a model drug.

2. Materials and methods

2.1. Reagents and chemicals

Furosemide (USP-grade, polymorph I) and LudipressTM were purchased from PREST (Buenos Aires). The chemicals employed were of analytical grade (Merck KgaA, Darmstadt, Germany). The *n*-butanol and acetone employed for the preparation of polymorphs II and III were distilled before use. Dissolution media were prepared in double distilled water.

2.2. Preparation of Furosemide polymorphs II and III

Both polymorphs were prepared as reported by Matsuda and Tatsumi (1990). In order to produce form II, excess of form I was added to *n*-butanol and the system was heated to dissolve the drug. Any excess solid was removed by vacuum filtration, the solution was placed under nitrogen and the polymorph crystallized as the *n*-butanol was evaporated. The crystals were collected by vacuum filtration and allowed to dry overnight, under reduced pressure at

room temperature. Finally, they were sieved through 50 and 70 mesh sieves, using for the next steps the solids that passed through the first sieve but not through the second.

Polymorph III was prepared by slowly evaporating a 5% solution FUR in acetone at room temperature, in a rotary evaporator under reduced pressure. The resulting crystals were collected, allowed to dry at room temperature overnight, and sieved through 50 and 70 mesh sieves. The polymorphs were characterized by FT-IR spectroscopy and thermomicroscopy.

2.3. Preparation of Furosemide-loaded capsules for dissolution analysis

Ludipress (12.5 g) and pure Furosemide polymorphs I, II and III (2.5 g) were weighed exactly, sieved through 50 and 70 mesh sieves, using for the next steps the solids that passed through the first sieve but not through the second. The solids were placed in a container and mixed in a mechanical shaker at 60 strokes/min until homogeneous. The mixing procedure was monitored by FT-IR spectroscopy by means of periodical sampling every 5 min after the first 20 min, until two spectra were congruent (30–45 min). Three batches of capsules (40 units per lot) were prepared by hand-filling size four hard gelatin capsules. A fourth batch was prepared by gently crushing and disintegrating commercial tablets (40 mg FUR, 200 mg Ludipress and 5 mg magnesium stearate) to powder, which was homogenized, sieved through 50 and 70 mesh sieves and encapsulated in the same way. The four batches complied with weight variation and content uniformity tests (USP Convention, 2007).

2.4. Equipment

The dissolution tests were performed with a Hanson SR8-Plus dissolution test station (Hanson Research, Chatsworth, USA). The pH values of the dissolution media were determined employing a model 125 Corning pH-meter (Corning, Inc., New York, USA) fitted with a Corning combined glass electrode. The amounts of drug dissolved were determined in 1.00 cm quartz cells, employing a Shimadzu UV-1601PC spectrophotometer (Shimadzu Corp., Kyoto, Japan) interfaced to a computer running Shimadzu's UV-Probe software v. 2.00. The infrared spectra were taken in a Shimadzu Prestige 21 FT-IR spectrophotometer (Shimadzu Corp., Kyoto, Japan) interfaced to a computer running IR Solution software. Samples, prepared as Fluorolube mulls, were held between two NaCl tablets. The thermomicroscopy studies were carried out with an Ernst Leitz 350 hot-stage microscope (Ernst Leitz, GmbH, Wetzlar, Germany), at a controlled heating rate of 10 °C/min in the range 30–200 °C.

2.5. Dissolution conditions

Since the capsules had a tendency to float, the studies were carried out in a test station configured with baskets as USP apparatus I (USP Convention, 2007; FDA, 2000), employing 900 mL of USP 30 dissolution medium of pH 2.2 (HCl-KCl), thermostated at 37 ± 0.5 °C; the baskets were rotated at 75 rpm. Twelve units were tested for each lot, run in two successive sets of six. During the experiments 3 mL aliquots were taken from each vessel at pre-specified times, without replacement; the samples were filtered and adequately diluted with dissolution media. The amount of dissolved FUR in the samples was determined by spectrophotometric measurement of their absorbances at 274 nm against a blank of dissolution medium and comparison with standard solutions containing known concentrations of FUR. Each dissolution profile was prepared with 17 time points, taking into account volume correc-

2.6. Data analysis

The computations were performed in Matlab v. 5.3 (Mathworks, Inc., Natick, MA), employing the previously described PCA–CR algorithm (Maggio et al., 2008). The Matlab scripts are freely available from the authors.

2.7. Principal component analysis. Theoretical background

Principal component analysis is a multivariate tool, useful to visualize representative features in multidimensional data, by reducing noise and data dimension; since its principles have been discussed elsewhere (Wold et al., 1987; Jolliffe, 2002), a short background is provided. Given matrix $\mathbf{X}_{(p \times t)}$, where each row contains t different pieces of information gathered from p objects, the mean centered data matrix $\mathbf{Xc}_{(p \times t)}$ can be obtained by subtracting the row vector containing the mean values of its columns [$\mathbf{Xm}_{(1 \times t)}$], from each row of the original matrix (\mathbf{X}).

The PCA algorithm performs a linear transformation of the set of random vectors x_i ($i = 1, \dots, p$) to a new set of vectors (w_i , where $i = 1, \dots, p$), the principal components (PCs). These PCs are orthogonal to each other and ordered according to their ability to explain variation of the data, so that the first few PCs retain most of the variation present in the original variables. The first PC is oriented in the direction on which the variance of the original data is maximized.

$$\mathbf{Xc}_{(p \times t)} = \mathbf{U} \cdot \mathbf{S} \cdot \mathbf{V}^T \quad (1)$$

When PCA is implemented as in Eq. (1), employing the singular value decomposition (SVD) algorithm, \mathbf{Xc} is factorized into the product of the orthogonal scores matrix of left singular vectors $\mathbf{U}_{(p \times t)}$, the diagonal matrix $\mathbf{S}_{(t \times t)}$ and the orthogonal loadings matrix $\mathbf{V}_{(t \times p)}$ (Manly, 1986). Matrix \mathbf{U} represents the projections of the data on the PCs space; therefore, similar samples are represented by similar scores; \mathbf{S} is a diagonal matrix which contains the singular values, which are the square roots of the eigenvalues associated to the corresponding eigenvectors, the PC's. The largest eigenvalues correspond to the dimensions that explain larger amounts of variance of the dataset. On the other hand, the loadings matrix \mathbf{V} contains a column-wise arrangement of the weights contributed to the PCs by the original variables (eigenvectors). Matrix \mathbf{T} , known as the weighed (un-normalized) score matrix, is the product between \mathbf{U} and \mathbf{S} ($\mathbf{T} = \mathbf{U} \cdot \mathbf{S}$) and totals information regarding data variation.

In the PCA–CR method, matrix \mathbf{X} contains 24 dissolution curves ($p = 24$), corresponding to the reference and test sets, each one taken at t time points. Therefore, each row vector of \mathbf{X} represents a dissolution curve.

3. Results and discussion

The central aim of this work was to develop a chemometric methodology able to probe the polymorphic identity of the active pharmaceutical ingredient from its dissolution profile. For this purpose, a principal component analysis with confidence regions (PCA–CR) comparison scheme was developed where the test sample could be compared with reference samples containing unequivocally characterized polymorphs of the studied active pharmaceutical ingredient.

Therefore, authentic samples of the FUR polymorphs were prepared and their dissolution characteristics were studied in order to find discriminating conditions. Then, the dissolution characteristics of capsules containing powdered tablets of a commercial formulation were compared with the aid of PCA–CR, with those filled with the polymorphs admixed with the excipients declared in the commercial product.

The use of capsules was preferred to the alternative strategy of preparing tablets, which entails the risk of compression-induced

polymorph conversion (Cheng et al., 2008), being a useful option in view of the lack of pharmacopoeial methods for dissolution testing of powders (Azarmi et al., 2007). The capsules served as convenient containers for the polymorphs in their mixtures with excipients and also for the tablet powders. This allowed standardization of dissolution factors related to the dosage form. In addition, sieved powders were used, in order to minimize the influence of particle size.

3.1. Characterization of the FUR crystalline polymorphs

Commercial bulk FUR, which was demonstrated to consist of form I by X-ray powder diffraction (Abdallah et al., 1989) and differential scanning calorimetry (Matsuda and Tatsumi, 1990), served as starting material for the preparation of polymorphs II and III. To ensure their identity, the individual polymorphic forms were analyzed by FT-IR spectroscopy, comparing the results with the data of Matsuda and Tatsumi (1990). Fig. 2 shows the corresponding spectra, taken in the 600–4000 cm^{-1} region. The main differences between the polymorphs were seen in the 3600–3000 cm^{-1} zone; these included the relative height of the peak at 3370 cm^{-1} , attributable to N–H stretching vibration of the secondary amine and the intensity of the 3420 cm^{-1} band, both increasing from polymorph I to form II and to polymorph III. The observed spectral features agreed with those reported.

The melting behaviour of forms II and III was also studied, employing hot-stage microscopy. Upon heating, characteristic morphological changes of the different crystals were observed, in complete agreement with the literature (Matsuda and Tatsumi, 1990).

3.2. Search for discriminating dissolution conditions

The range of solubility differences between different polymorphs is typically only 2–3-fold due to relatively small differences in free energy (Singhal and Curatolo, 2004) and it is known that the dissolution test becomes more discriminating when the solubility of the drug and its rate of dissolution are low (Nicklasson and Magnusson, 1985). Akbuğa and Gürsoy (1987) found that dissolution media of pH between 4.0 and 5.0 were convenient to distinguish between properly and poorly formulated FUR tablets and the USP 30 dissolution test of FUR tablets employs phosphate buffer pH 5.8 (USP Convention, 2007).

Therefore, the dissolution behaviour of the different reference polymorphs of FUR, was studied in official dissolution media of pH 2.2, 4.1 and 5.8 (USP Convention, 2007). The profiles complied with the requirements that their coefficient of variation was not more than 20% at the earlier time points, and not more than 10% at the other time points (FDA, 2000). Interestingly, as shown in Fig. 3, at pH 5.8 the dissolution behaviour of the three polymorphs was highly similar, probably as a consequence of the fact that deprotonation of the carboxylic acid moiety of FUR facilitates dissolution. However, differences between the forms became evident at lower pH values. Polymorph I exhibited the greatest extent of dissolution at pH 2.2 and 4.1, differentiating itself from its congener forms II and III. On the other hand, the latter pair of polymorphs showed dissolution profiles of approximately the same amplitude. In order to ensure selection of the most discriminating medium, the dissimilarity between the dissolution profiles of these two polymorphs was evaluated.

The angle between two vectors is a measure of how closely related they are, the wider the angle the worse their relationship (Gargallo et al., 1996; Kuragano and Yamaguchi, 2006); therefore, a vectorial comparison between the profiles of the polymorphs II and III, at pH 2.2 and 4.1 was carried out. Determination of the angle (θ) between the profiles was performed according to Eq. (2), where A and B are the dissolution profile vectors of polymorphs II and III,

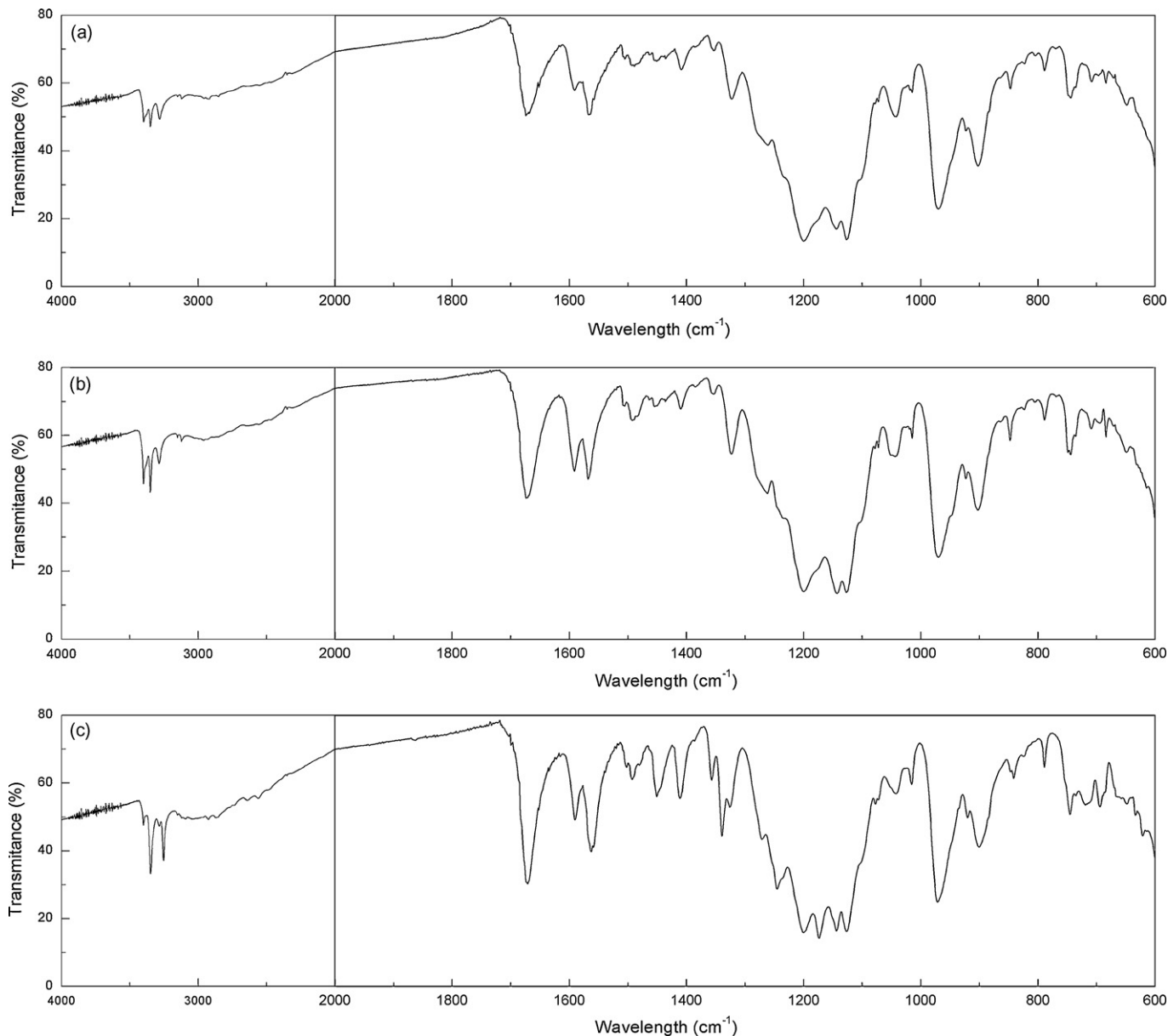


Fig. 2. FT-IR spectra of the three crystalline polymorphs of FUR, as Fluorolube mulls. (a) Polymorph I; (b) polymorph II and (c) polymorph III.

respectively, and $\|A\|$ and $\|B\|$ represent their corresponding norms.

$$\Theta = \arccos \frac{A \cdot B}{\|A\| \|B\|} \quad (2)$$

This revealed that at pH 2.2, the angle was 6.7° , while at pH 4.1, a value of $\Theta = 1.2^\circ$ was obtained. The wider angle observed between the profiles at the lower pH indicated the latter as the most discriminating condition; therefore, pH 2.2 was selected for development

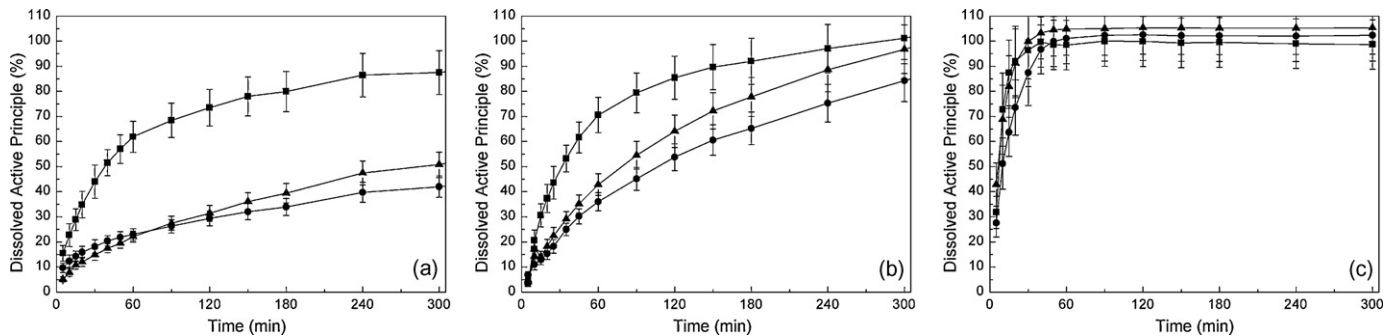


Fig. 3. Dissolution profiles of the reference Furosemide polymorph mixtures. (■) = Polymorph I; (▲) = polymorph II and (●) = polymorph III, in 900 mL of USP dissolution media of different pH values. (a) pH 2.2; (b) pH 4.1 and (c) pH 5.8.

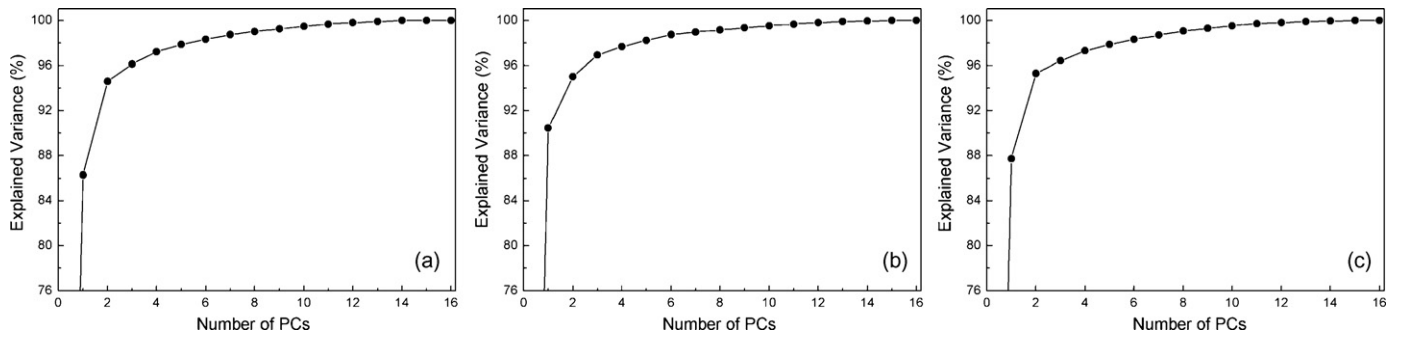


Fig. 4. Percentage of explained variance against number of PCs for the different polymorphic forms of FUR. (a) Form I; (b) form II and (c) form III.

of the proposed method. This condition is also bio-relevant, since when used orally FUR is mainly absorbed from the stomach. The low solubility of the drug in this medium becomes the rate-determining step for its absorption and bioavailability (Hammarlund et al., 1984).

3.3. PCA-CR study of the dissolution behaviour of the different polymorphs

The PCA-CR method for comparing dissolution profiles has five steps, consisting in (a) outlier detection and removal among the sets of data being compared; (b) construction and mean-centering of a single matrix containing the dissolution curves of the batches being compared and selection of the relevant number of PCs that allow proper reconstruction of the original data matrix; (c) graphing of a scores plot; (d) building the confidence region around the scores of the batch taken as reference and (e) taking a decision with regard to similarity of the dissolution profiles being compared, according to an established rule (Maggio et al., 2008).

In order to ensure data quality, absence of outliers was assessed employing Hotelling's test, where Mahalanobis distances were calculated for the 12 dissolution curves in each set and compared with the corresponding χ^2 [99% confidence level and 17 (number of data points per curve) degrees of freedom]. Calculations were carried

out according to Eq. (3), where μ_x is the mean curve (dissolution profile) of the dataset and S_{XX}^{-1} is inverse of the covariance matrix (S_{XX}) of the data. No outlier data were found.

$$12 \times (x - \mu_x)^T S_{XX}^{-1} (x - \mu_x) < \chi_{0.99, 17}^2 \quad (3)$$

$$X_{c(p \times t)}^* = U^* S^* V^{*T} [r \leq p] \quad (4)$$

Next, the optimum number of principal components (r) were obtained, by setting the requirement that they should explain at least 95% of the variance in the reconstructed data matrix X^*c (Eq. (4)). The use of two PCs allowed compliance with this condition (Fig. 4).

Therefore, for comparison purposes, the values of the first two weighed scores contained in the columns of matrix T (PCs) of their dissolution profiles were plotted, as shown in Fig. 5. A 99% confidence region based on Hotelling's equation (Eq. (3)) was drawn for each reference lot, according to Eq. (5), where d_1 and d_2 are eigenvalues of the covariance matrix S_{XX} of the data; d_1 and d_2 are related to the lengths of the axes of the ellipses, defined by $(9.21 \times d_1)^{0.5}$ and $(9.21 \times d_2)^{0.5}$, respectively ($9.21 = \chi_{0.99, 2}^2$). On the other hand, vectors w_1 and w_2 provide information about the orientation of the ellipse. These are elements of matrix $W = B(x - \mu_x)$, where the rows

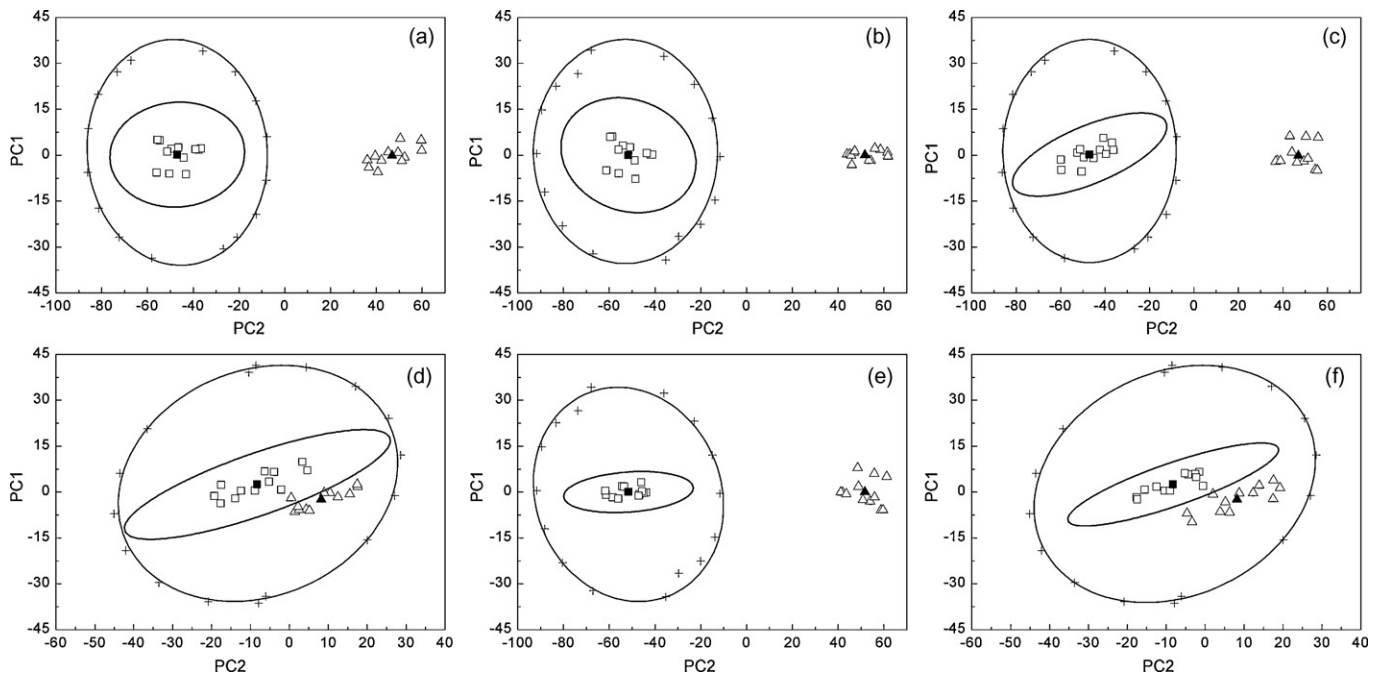


Fig. 5. PCA-CR comparisons [reference (\square) and test (Δ)] between batches containing FUR polymorphic forms I, II and III. (a) I–II; (b) I–III; (c) II–I; (d) II–III; (e) III–I and (f) III–II. The 99% confidence level ellipses (—) and the $f_2 = 50$ ellipses (---), obtained by the bootstrapping technique, are shown. The average coordinates of the first two PCs of the reference (\bullet) and test (\blacktriangle) batches are also indicated.

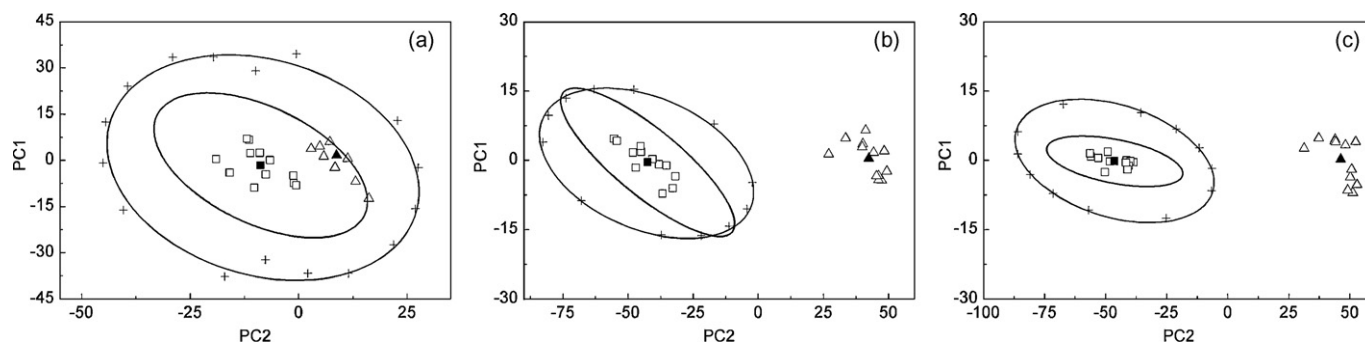


Fig. 6. PCA-CR comparisons [reference (\square) and test (Δ)] between batches containing FUR polymorphic forms I, II and III, and a test product (Test). (a) I-Test; (b) II-Test and (c) III-Test. The 99% confidence level ellipses (—) and the $f_2 = 50$ ellipses (---), obtained by the bootstrapping technique, are shown. The average coordinates of the first two PCs of the reference (\blacksquare) and test (\blacktriangle) batches are also indicated.

of **B** are eigenvectors of matrix S_{XX} .

$$P \left[\left(\frac{w_1^2}{9.21d_1} - \frac{w_2^2}{9.21d_2} \right) < 1 \right] = 0.99 \quad (5)$$

In order to take into account manufacturing variations and data variability (minor differences in particle characteristics, composition of the test and reference batches, etc.), the classification criterion adopted was that at least 80% of the units of the test batch should be included in the 99% confidence region of the reference polymorph.

When the score plots corresponding to all of the possible comparisons among the three reference polymorphs in their compounded samples were analyzed (Fig. 5), their dissimilarity was clearly evident, despite the closeness between the average scores of polymorphs II and III (Fig. 5d and f). This confirmed the ability of PCA-CR to discriminate among the polymorphic forms of FUR based on their dissolution characteristics. However, when the test batch containing the powdered commercial product (prepared with the polymorph I of FUR) was compared with the three reference batches through analogous score plots (Fig. 6), it was observed that similarity could be achieved only with the reference of polymorph I (Fig. 6a). Hence, it can be inferred the presence of polymorph I in the test lot.

On the other side, the f_2 similarity factor shown in Eq. (6) (Moore and Flanner, 1996) is being recommended by the FDA and other regulatory bodies as a means to test couples of batches for similarity from their corresponding dissolution profiles. Test (T) and reference (R) batches are considered similar if values of $f_2 \geq 50$ are obtained, when compared at t time points.

$$f_2 = 50 \log \left[\left(1 + \left(\frac{1}{t} \right) \sum_{t=1}^t (R_t - T_t)^2 \right)^{-0.5} \times 100 \right] \quad (6)$$

Employing a bootstrapping technique, (Efron and Tibshirani, 1986, 1993), the coordinates of ellipses encircling the $f_2 \leq 50$ area were obtained, as shown in Fig. 5. For that purpose, the mean vector data (dissolution profiles) of the reference batches [$x_{(1 \times t)}$] were transformed into new vectors [$x_{(1 \times t)}^*$] by replacement of some of their items with artificial data able to produce f_2 values near 50, and the procedure was repeated a number of times.

When employed to compare the dissolution characteristics of the different polymorph standards, the estimations based on bootstrap-mediated f_2 calculations were not able to differentiate between FUR forms II and III (Fig. 5d and f). Moreover, similarity was also evident from the estimation of f_2 between the dissolution profiles of both polymorphs at this pH ($f_2 = 58.3$). Nevertheless, the bootstrap-based f_2 estimator also assigned polymorph I to the test sample (Fig. 6a), although the corresponding $f_2 = 50$ ellipse covered

a greater area than that of PCA-CR. The superior ability of PCA-CR methodology to differentiate among the polymorphic forms of FUR is probably a result of its sensitivity to shape and size of the dissolution curves. An additional advantage is that PCA-CR is able to perform polymorph assignment with a given degree of statistical confidence.

4. Conclusions

Chemometric techniques are powerful tools for compressing multivariate data into few variables and unveiling hidden trends. Employing Furosemide as model drug, it has been shown that principal component analysis with confidence regions (PCA-CR) is capable of providing information regarding the polymorphic form in a pharmaceutical formulation. This strategy was based on comparison of the dissolution behaviour of a test batch of Furosemide with those containing standards of the polymorphs of the drug. Discriminating conditions for the test were achieved at pH 2.2 (HCl-KCl solution).

Results of PCA-CR were compared with those acquired by application of the f_2 similarity factor, both agreeing in the conclusion reached for the test sample. Interestingly, however, the $f_2 = 50$ ellipses, obtained by bootstrapping techniques, enclosed a much greater area than the corresponding 99% confidence regions drawn around the PC scores. This resulted in the f_2 ellipses not being able to differentiate between FUR polymorphs II and III.

The PCA-CR method, which represents a simple approach to probing polymorph form in a pharmaceutical product, is inexpensive and of easy implementation, providing results with statistical support.

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