Article

Application of a New Strategy of Validation Based on "β,γ-Content Tolerance Interval" for Checking the Chiral Chromatography Method for Quantification of the Chiral Impurity of Levofloxacin

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A simple and accurate chiral high performance liquid chromatography (HPLC) method was developed for determination of Levofloxacin and its chiral impurity (*R*)-enantiomer. The chiral impurity Levofloxacin separation method was optimized by Box-Behnken design and validated by an innovative analytical approach recently developed in our laboratory. This last approach as well as uncertainty estimation consists of building a graphical decision-making tool, based on the β,γ-content tolerance interval, called the uncertainty profile. The proposal approach applied to the optimized chiral separation method of Levofloxacin shows its fitness.

Keywords: Levofloxacin, experimental design, uncertainty profile, β,γ-content tolerance interval, measurement uncertainty

Introduction

Recently an original approach based on β,γ-content tolerance interval and uncertainty profile has been introduced. This approach has the advantages that it can facilitate the analytical validation by providing a decision tool based on the uncertainty profile, and allows a good estimate of measurement uncertainty using validation data without additional experiment. An enantiomeric separation using chiral chromatographic method for the determination and quantification of chiral impurity of Levofloxacin was used to illustrate the applicability and flexibility of this approach.

A large number of drugs containing an asymmetric center, which is called chiral drug, are used in the pharmaceutical industry. Although it is well established that some enantiomers can have different biological and pharmacological activities, which in some cases can have side effects or even toxic effect,[1] they are still administered as racemic mixture. It is why in the recent years, many chiral separation methods were developed.[1-8]

Levofloxacin (Figure 1a) is a chiral fluorinated carboxy quinolone a pure (−)-(*S*)-enantiomer of the racemic, drug substance ofloxacin. Its antibacterial activity is twofold stronger than that of the *R*-isomer ofloxacin (Figure 1b).[2,9]

As in all synthesis routes used to produce Levofloxacin may contain some quantity of its chiral impurity (*R*-isomer of ofloxacin), the establishment of substantial separation method for the two enantiomers to control the chiral purity of the drug would be mandatory.[1-4]

In the literature, different reversed high performance liquid chromatography (HPLC) methods using ligand-exchange, for
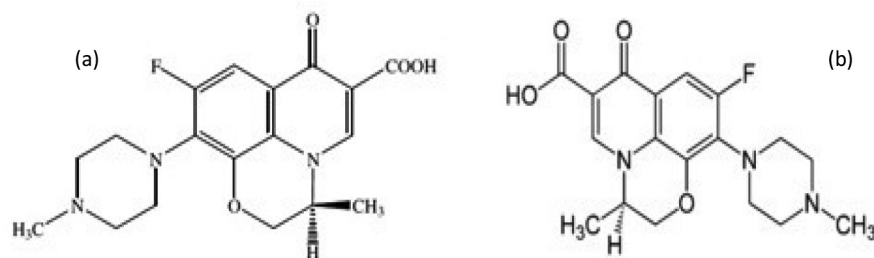
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Figure 1. Levofloxacin (a) and (b) *R*-isomer of ofloxacin (chiral impurity of Levofloxacin).

ofloxacin enantiomer determinations were reported.[1-4] Chiral capillary electrophoresis using various cyclodextrins as chiral selector was also reported.[5-7] So far in our knowledge, only one HPLC method has been reported using chiral column for the determination of ofloxacin enantiomers.[8] However, none one of the cited method has used an experimental design for method optimization.

The aim of this study was to develop and validate a simple chiral HPLC method for determination of Levofloxacin and its chiral impurity. The development and optimization was done by using three-factorial Box-Behnken design and the analytical validation was applied using an innovative approach, recently developed in our laboratory;[10-13] the new approach consists of building a graphical decision-making tool, called the uncertainty profile based on the β,γ-content tolerance interval. Further, the method will be applied in routine analysis for the quantification of chiral impurity of Levofloxacin and uncertainty evaluation.

Experimental

Chemicals and solvents

Ofloxacin (99.6%) and Levofloxacin (101.5%) standards were obtained from pharmaceutical industries like SANOFI (Casablanca, Morocco). Methanol, hexane, heptane, ethanol of HPLC grade were from Sigma Aldrich (Taufkirchen, Germany). Phosphoric acid (85%) and triethylamine were obtained from Scharlau (Barcelona, Spain).

Instrumentation and chromatographic conditions

The chromatography system consisted of Waters (Waters, Milford, MA, USA) 2695 pumps, auto sampler, automatic injection and Waters 2996 photodiode-array detector (PDA). Separation was carried on Chiralcel OD-H column (250 × 4.6 mm) packed with 5-μm silica gel coated by Cellulose *tris*-3,5-dimethylphenylcarbamate (Osaka, Japan) and maintained at 35 °C. The mixture of hexane solution and methanol plus ethanol solution in the ratio of (74:26 v:v) containing 0.45% of phosphoric acid and 0.05% of triethylamine was used as the mobile phase at a flow rate of 1.2 mL min^{-1}. The wavelength of detection and the injection volume were set at 294 nm and 50 μL, respectively. Data were acquired and processed with Empower chromatography manager software.

Resolution solution for method optimization

A stock solution of ofloxacin standard containing Levofloxacin (0.05 mg mL^{-1}) and chiral impurity of Levofloxacin (0.05 mg mL^{-1}) was prepared in methanol. This solution was filtered through 0.45 μm Millipore filter (polyethylene, PE) before chromatographic analysis and it was used for an optimization study.

Standard solution for method validation

A stock solution containing 0.5 mg mL^{-1} of chiral impurity of Levofloxacin was prepared in methanol. Further dilutions were performed to obtain five working calibration standards at concentration levels (0.0005, 0.00125, 0.0025, 0.005, 0.01 mg mL^{-1}).

Validation standards were prepared by stock dilution to give the same concentration levels the calibration standards adding 0.5 mg mL^{-1} of active pharmaceutical ingredient of Levofloxacin as a matrix.

Experimental design

Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for the improvement and optimization of complex processes. The main advantage of RSM is its ability to reduce number of experimental trials needed to evaluate multiple parameters and their interactions to provide sufficient information for statistically acceptable results. In addition, it is commonly used to find the optimum chromatographic conditions for separation of drug compounds.

Three classes of response surface designs can be used, central composite, Doehlert and Box-Behnken.

Table 1. Factors and its corresponding levels selected for the Box-Behenken design

Factor	Unit	Level (−)	Medium	Level (+)
Mixture of methanol plus ethanol, X_1	%	25	30	35
Temperature of the column oven, X_2	°C	25	30	35
Flow rate, X_3	mL min^{-1}	0.8	1.0	1.2

Column, volume injection and detection wavelength were maintained constant.

Box-Behnken design is preferable to the central composite and Doehlert designs not only because it requires fewer test runs but also because it is rotatable. Indeed, Box-Behnken designs do not contain any points at the extremes of the cubic region created by the two-level factorial.[14]

In this work, we have used a three-level and three-factorial Box-Behnken experimental design to estimate the effects of the selected independent variables on the responses, to optimize the separation between Levofloxacin and its chiral impurity in short time. This design is appropriate for exploration of quadratic response surface and for construction of second order polynomial models (equation 1).[14-17]

The statistical model used for this design is of the following form:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + e \qquad (1)$$

where Y is the selected response, which calculated by the model, X_1, X_2 and X_3 are the factor studied, b_1, b_2 and b_3 are linear effects, b_{12}, b_{13} and b_{23} are the interaction effects and b_{11}, b_{22} and b_{33} are quadratic effects, e is an error term.

Factors studied in the Box-Behnken experimental design were: mixture of methanol plus ethanol (X_1) to achieve a good chiral separation, temperature of the column oven (X_2) which can influence the viscosity of the mobile phase, thus affect the migration of the solute in the column and flow rate (X_3) to perform a suitable retention time (Table 1). The selected responses were: Rs (Y_1) resolution between Levofloxacin and chiral impurity and Rt (Y_2) retention time of the chiral impurity. Table 2 shows the levels combination for the factors studied in each experimental trial.

Method validation

Validation is a very important step in analytical method, it allows ensuring the results obtained in terms of reliability. For a long time, the classical validation that treats separately the two errors such as the bias and random error to make the decision was applied. However, statistical processing used by this approach presents some ambiguities, especially in decision-making and interpretation of results.

Table 2. Levels combination for the factors and retention time for each experimental trial

Experience number	Mobile phase / %	Temperature / °C	Flow / (mL min^{-1})
1	25	25	1.00
2	35	25	1.00
3	25	35	1.00
4	35	35	1.00
5	25	30	0.80
6	35	30	0.80
7	25	30	1.20
8	35	30	1.20
9	30	25	0.80
10	30	35	0.80
11	30	25	1.20
12	30	35	1.20
13	30	30	1.00
14	30	30	1.00
15	30	30	1.00

Selectivity

The selectivity of the method is a very important step that can be checked by, the existence or absence of any interference with the chiral impurity of Levofloxacin, and the matrix.

Response function

The relationship between the response and the analytical concentration was determined on the basis of the calibration standards. And it was characterized by a function called response function, which is a critical step in the analytical validation. Indeed, the series of calibration standards prepared using material reference help to generate many regression models for calibration, e.g., the linear regression through 0, the linear regression model, the weighted 1/X or 1/X^2 linear models, the quadratic regression model, the linear regression after square root transformation data, the linear regression after logarithm transformed data.[18-28] We will then select the most suitable model that allows a good inverse prediction. This inverse prediction performed, via the selected model,

allowed giving the retrieved concentrations, the relative bias and the relative standard deviation (RSD) precision for each concentration level.

Estimation of precision

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.[18-28] In the present study, the repeatability and intermediate precision can be evaluated at each level of concentration using one way analysis of variance. During the analytical method validations, the experimental design involves to make measurements at different days, with replicate determinations within each day. A statistical model to describe the measured values is given by equation 2:

$$Y_{ij} = \mu + b_i + e_{ij}; \; j = 1,2,\ldots,n; \; i = 1,2,\ldots,a \tag{2}$$

where Y_{ij} denotes the jth replicate observation corresponding to i^{th} run, μ is an unknown general mean, b_i's represent random effects. and e_{ij}'s represent error terms. It is assumed that b_i's and e_{ij}'s are all independent having the distributions b_i ca. $N(0, \sigma_b^2)$ and e_{ij} ca. $N(0, \sigma_b^2)$. Thus and σ_b^2 and σ_e^2 represent the two variance components in the model.

We define:

$$\overline{Y} = \frac{1}{an} \sum_{i=1}^{a} \sum_{j=1}^{n} Y_{ij}, \; \overline{Y}_i = \frac{1}{n} \sum_{j=1}^{n} Y_{ij}, \; SS_b = n \sum_{i=1}^{a} \left(\overline{Y}_i - \overline{Y} \right)^2$$
$$\text{and } SS_e = \sum_{i=1}^{a} \sum_{j=1}^{n} \left(Y_{ij} - \overline{Y}_i \right)^2 \tag{3}$$

where $\overline{Y}$ is the mean of the back calculated concentration. And $\overline{Y}_i$ is the mean of the back calculated concentration at i level. SS_b present square of standard deviation inter-series and SS_e present square of standard deviation intra-series.

Based on the model in equation 2, we can identify the expected mean squares (equation 4):

$$MS_b = \frac{SS_b}{a-1} \text{ and } MS_e = \frac{SS_e}{a(n-1)} \tag{4}$$

where MS_b and MS_e are the mean square model and the mean square error, respectively.

The ANOVA estimators of σ_b^2 and σ_b^2 are given by:

$$S_b^2 = \frac{1}{n} \left(MS_b - MS_e \right) \text{ and } S_e^2 = MS_e \tag{5}$$

where S_b and S_e are standard deviation inter-series and standard deviation intra-series, respectively.

If $MS_e < MS_b$, the repeatability and intermediate precision will be, respectively (equation 6):

$$S_r^2 = MS_e \text{ and } S_{FI} = \sqrt{S_e^2 + S_b^2} \tag{6}$$

If not, $S_b^2 = 0$ and $S_e^2 = S_T^2$; where, S_T^2 was the total variance of the set of measures.

Estimation of trueness

The trueness of an analytical procedure (or bias), at each concentration level, is obtained by calculating the difference between the introduced concentrations mean ($\hat{\mu}_m$) and the calculated concentrations mean ($\overline{x}_m$). The bias can be expressed in absolute or relative terms (equation 7) or in recovery terms (equation 8),[18-28] as follows:

$$\text{Bias (\%)} = \frac{\overline{x}_m - \hat{\mu}_m}{\hat{\mu}_m} \times 100 \tag{7}$$

$$\text{Recovery (\%)} = \frac{\overline{x}_m}{\hat{\mu}_m} \times 100 \tag{8}$$

Uncertainty profile

In this study, an innovative approach, recently developed by our laboratory,[10-13] was used which allows at the same time examination of the validity of analytical procedures as well as estimation of the uncertainty of chemical measurements without additional experiments. It consists of building a graphical decision-making tool, called the uncertainty profile. Our proposal allows a good assessment of measurement uncertainty through an innovative formula, based on the β,γ-content tolerance interval.

The validation strategy based on the uncertainty profile can be achieved through the following steps:
(*i*) choice of the appropriate acceptance limits taking into account the intended use of the method;
(*ii*) generate all possible calibration models using the calibration data;
(*iii*) calculation of the inverse predicted concentrations of all validation standards according to the selected calibration model;
(*iv*) compute the two-sided β-content γ-confidence tolerance intervals for each level, according to one of three approaches proposed below;
(*v*) determination of the uncertainty for each level using equation 9 below;
(*vi*) construct the uncertainty profile according to equation 9 below and make 2D-graphical representation results for the acceptability and uncertainty limits;

(*vii*) compare the interval of uncertainty (L, U) to the acceptance limits (−λ, λ);

(*viii*) if (L, U) falls totally within (−λ, λ), the method is accepted; otherwise, the method is not valid.

The computation of the two sided β-content tolerance intervals for balanced one-way random effects models using Mee's method was carried out according to the method described by Saffaj and Isshane[10] And which its applicability and its performance have been demonstrated to validate analytical and bioanalytical methods.[29,30]

The formula to assess the uncertainty at each level of concentration was demonstrated as follows:

In accordance with the LGC/VAM protocol[28] and the recommendations of the ISO/DTS 21748 guide, a basic model for the uncertainty of the measurand Y can be expressed by (equation 9):

$$u^2(Y) = S_R{}^2 + u^2(\hat{\delta}) + \Sigma C_i{}^2 u^2(x_i) \tag{9}$$

where S_R is the reproducibility standard deviation, $u(\hat{\delta})$ is the uncertainty associated with the bias of the method, and $\Sigma C_i{}^2 u^2(x_i)$ is the sum of all of the effects due to other deviations.

The previous accuracy profile and validation data concept used by Feinberg[23] to estimate the measurement uncertainty, the third term of equation 9 was ignored. However, uncertainty can be expressed by the following equation (equation 10):

$$u^2(Y) = S_R{}^2 + u^2(\hat{\delta}) \tag{10}$$

The accuracy profile can be built using the β-expectation tolerance interval, according to Mee's method.[31] This interval is equal to equation 11:

$$\bar{Y} \pm t(\nu)k\hat{\sigma}_M \tag{11}$$

Thus, one can verify that equation 12:

$$u^2(Y) = k^2 \hat{\sigma}^2{}_M \tag{12}$$

And the mathematical model that brings the uncertainty and the tolerance interval is given by equation 13:

$$\bar{Y} \pm t(\nu)u(Y) \tag{13}$$

Note that: $\hat{\sigma}_M = S_R$

By virtue of equation 13 we can write that equation 14:

$$U = \bar{Y} + t(\nu)u(Y) \tag{14}$$

and:

$$L = \bar{Y} - t(\nu)u(Y) \tag{15}$$

Finally, the uncertainty can be expressed as equation 16:

$$u(Y) = \frac{U - L}{2t(\nu)} \tag{16}$$

where U is the upper β-content tolerance interval, L is the lower β-content tolerance interval, t(ν) is the (1 + γ)/2 quantile of Student's *t*-distribution with ν degrees of freedom. For balanced data, ν can be estimated by the Satterthwaite formula.[32]

Finally, in order to build the uncertainty profile, we have used the following formula equation 17:

$$|\bar{Y} \pm ku(Y)| < \lambda \tag{17}$$

where k is a coverage factor. The choice of the factor k is based on the level of confidence desired. For an approximate level of confidence of 95%, k = 2. $\bar{Y}$ is the estimate of the mean results, λ is the acceptance limits.

The uncertainty profile can also be expressed by equation 18:

$$|Bias \pm ku(Y)| < \lambda \tag{18}$$

Results and Discussion

ANOVA result and regression model with the aid of Box-Behenken design

According to analysis of variance (ANOVA), the regression model is significant for the two response respectively resolution Rs and retention time Rt, this is evident from the Fisher's *p*-value (6.6 and 16.6) and a low probability value ($p < 0.05$) for the two response.

The determination coefficient (99.7%) and (99.98%) for Rs and Rt respectively confirms the adequacy of model. The value suggests that the model could predict 95.6% of the variability in Rs and 99.8% for Rt.

The second-order polynomial equation illustrates the relationship of the three variables (equations 19 and 20).

$$Y1 = 2.44 - 0.33X_1 - 0.27X_2 - 0.14X_3 - 0.11X_1^2 + 0.04X_3^2 + 0.07X_1X_2 \tag{19}$$

$$Y1 = 12.88 - 3.85X_1 - 1.48X_2 - 2.69X_3 - 0.15X_1^2 + 0.32X_3^2 + 0.73X_1X_2 + 0.81X_1X_3 \tag{20}$$

Table 3. Factor effects and associated *p*-values results for Box-Behnken design

Term	Resolution (Rs)		Retention time (Rt)	
	Estimate	*p*-value / %	Estimate	*p*-value / %
X_1	−0.331	< 0.01[a]	−3.851	< 0.01[a]
X_2	−0.278	< 0.01[a]	−1.486	< 0.01[a]
X_3	−0.145	< 0.01[a]	−2.696	< 0.01[a]
X_1X_2	0.074	0.614[a]	0.73	< 0.01[a]
X_1X_3	0.015	40.5	0.81	< 0.01[a]
X_2X_3	0.04	5.4	−0.039	30.9
X_1X_1	−0.116	0.128[a]	−0.156	0.835[a]
X_2X_2	−0.001	93.5	−0.014	72.1
X_3X_3	0.045	4.13[a]	0.329	0.0522[a]
Mean square of regression	0.1947		22.1957	
Mean square of residues	0.001		0.0049	
Mean square of the error	0.0001		0.0014	
Lack of fit (Fisher's value)	6.6		16.6	
R^2	0.997		0.9998	
R^2 adjusted	0.991		0.9996	

[a]Presents significant effect.

An independent factor had effect on a given response when it had a *p*-value < 0.05, and non-significant terms were eliminated from the model postulated a priori (Table 3).

Only significant coefficients with *p*-value < 5% are included. From the results illustrated in Table 3, it was concluded that proportion mixture of methanol plus ethanol (X_1), temperature of the column oven (X_2) and flow rate (X_3) were important significant factors affecting the two responses. Quadratic terms also created important effect, X_1^2 and X_3^2 present significant effect on investigated Rs and Rt. Factor interaction X_1X_2 had significant effect on measured responses (Rs and Rt), and X_1X_3 affect only Rt response.

Optimization of chromatographic conditions

ANOVA were verified before performing response surface methodology. Indeed, from the result presented in Table 3, since the variation of regression is greater than the variation of residues and the lack of fit is greater than the error, therefore the ANOVA is verified and the model is considered valid.

Response surface methodology (RSM) plays a very critical role in efficiently exploring the optimal values of explanatory variables. As a function of two factors, three dimensional response surfaces and their corresponding contour plots are more helpful in understanding both the main and the interaction effects of these two factors,

maintaining all other factors at fixed levels. They can be used to describe and examine the regression equations in a visualized way to reflect the effects of experimental variables on the required response.[33]

The variation of the response was correctly related to the variation of the factors. Three dimensional plots for the measured responses were performed, based on the model polynomial functions to assess the change of the response surface.

Figures 2a-2d show the effect of proportion mixture of methanol plus ethanol and temperature of the column oven on resolution and retention time at a constant flow rate of 1 mL min⁻¹, it is apparent from the figure that the resolution Rs and retention time Rt increased with decreasing the proportion of methanol plus ethanol and the temperature of the column oven. Figures 2b-2e show the effect of proportion mixture of methanol plus ethanol and flow rate on resolution and retention time at a constant temperature of column oven of 30 °C, like the last observation, the resolution Rs and retention time Rt increased with decreasing the proportion of methanol plus ethanol and the flow rate. Figures 2c-2f show the effect of temperature of column oven and flow rate at a constant proportion mixture of methanol plus ethanol of 30%, in that figure we remark that the resolution Rs and retention time Rt increased with decreasing the flow rate and the temperature of the column oven.

In a chromatographic method, the resolution and retention time are the most important parameters which characterize a chromatogram, the resolution indicate if the method is suitable to separate different analytes in a mixture solution, in our study we have to separate two enantiomers which present the same physicochemical characteristics, also their separation are not easy, that is is why this parameter was selected as an optimization criteria to ensure that the chosen method will be able to separate and quantify the Levofloxacin and its chiral impurity in the routine application. Afterwards, we try to have a short time for this chromatographic analysis, therefore we have chosen retention time of the chiral impurity like a second criteria of optimization.

Our objective in the optimization stage is to implement a chromatographic method that quantifies the chiral impurity of Levofloxacin in the active pharmaceutical ingredient, in a short time with a good separation between the peaks. Before continuing, it was important to identify some important criteria of optimization, indeed in the first hand minimum retention time of *R*-isomer (chiral impurity of Levofloxacin) in order to save acquisition time and the amount of solvents used in the mobile phase and in the other hand maximum resolution between the Levofloxacin and its *R*-isomer for the purpose of a good quantification of
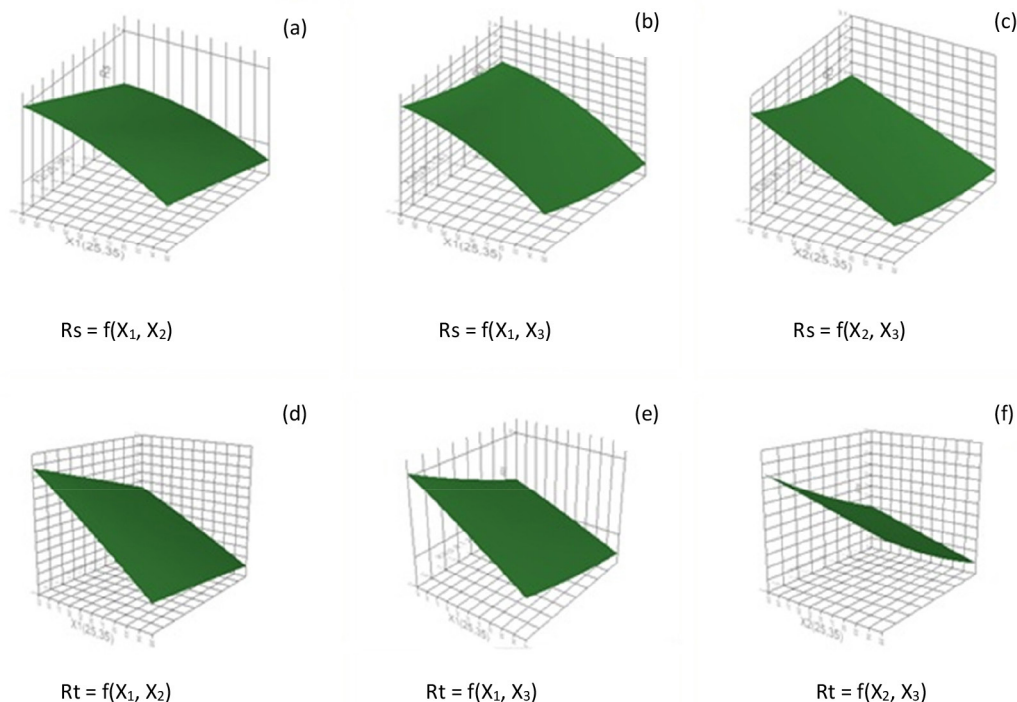
Figure 2. Responses surfaces related to proportion mixture of methanol plus ethanol (X_1), temperature of the column even (X_2) and flow rate (X_3) on the responses: resolution Rs and retention time Rt, using Box-Behnken design. (a,d) Show the effect of X_1 and X_2 on Rs and Rt; (b,e) show the effect of X_1 and X_3 on Rs and Rt; (c,f) show the effect of X_2 and X_3 on Rs and Rt.

the chiral impurity in the active pharmaceutical ingredient. A graphical method was used for global optimization which are based on contour overlay plots (Figure S1 (in the Supplementary Information (SI) section). These plots depict the alteration of all selected responses against two independent factors, whilst keeping the rest at constant level. The optimum experimental conditions were defined by the intersection between resolution (red curve) and retention time (blue curve).

The acceptable retention time and resolution can be established as follows: Figure S1a (in the SI section) account for portion mixture of methanol plus ethanol to be in the range 26-27% and temperature of column oven to be in the range 25-27 °C (Figure S1b in the SI section), account for proportion mixture of methanol plus ethanol to be in the range 26-27% and flow rate to be in the range of 1.1-1.2 mL min^{-1} (Figure S1c in the SI section) account for temperature of column oven to be in the range 25-26 °C and flow rate to be in the range of 1.1-1.2 mL min^{-1}.

Eventually, the optimal conditions for the separation and identification of *R*-isomer (chiral impurity of Levofloxacin) using Box-Behnken design were obtained with fallowing mobile phase: hexane and mixture of methanol plus ethanol (74:26 v:v) with 1.2 mL min^{-1} of flow rate and temperature of column oven of 25 °C. The chromatogram obtained from the above conditions is shown in Figure S2 (in the SI section). Under the described experimental conditions, the

values of retention time of *R*-isomer were 13.675 min and the resolution between Levofloxacin and *R*-isomer was 2.8. It is apparent that Box-Behnken statistical design was reliable and effective in determining the optimum conditions.

Method validation

After the optimization process, it is necessary to demonstrate that the analytical method provides accurate quantification results. This is carried out through a method validation. In this study the quantification of the chiral impurity of Levofloxacin was validated by applying the concept of uncertainty represented by an uncertainty profile.

Selectivity

It was first thought necessary to verify the absence of any interference at the retention time of the chiral impurity of Levofloxacin. For this, the selectivity of the method was evaluated. The chromatographic method conferred good selectivity for the *R*-isomer contained in active pharmaceutical ingredient of Levofloxacin. Indeed, the chromatogram overlay of the mixture of the Levofloxacin and *R*-isomer standard, active pharmaceutical ingredient preparation, mobile phase solution and blank solution showed no endogenous peak interference (Figure 3). All the analytes were well resolved under the optimal conditions.
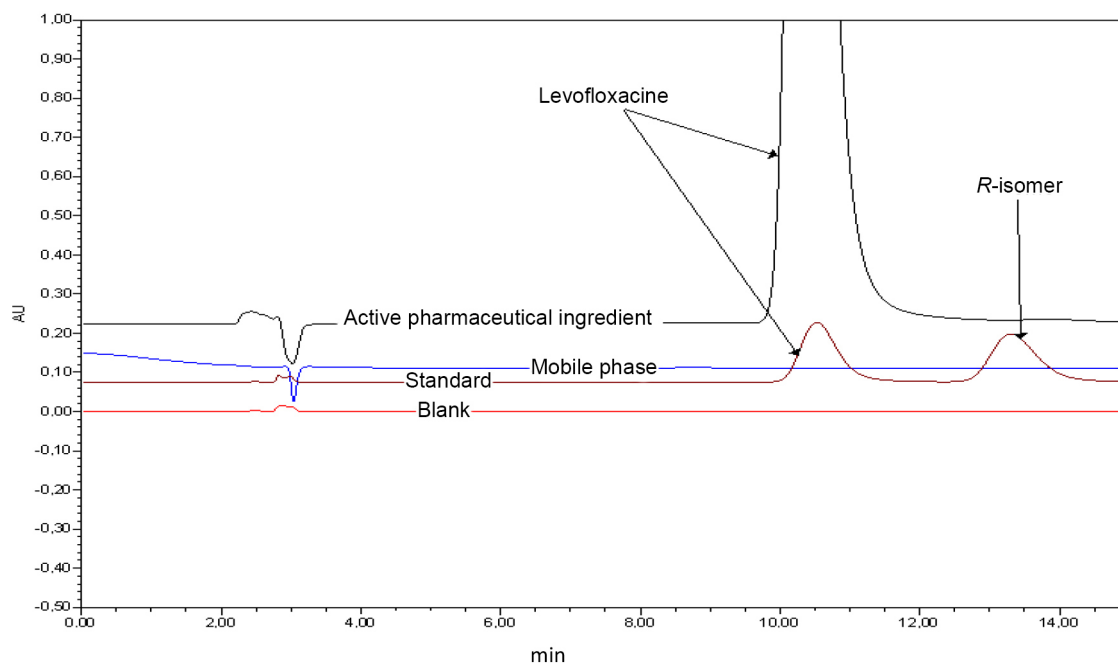
Figure 3. Chromatograms of the mixture of the Levofloxacin and *R*-isomer standard, active pharmaceutical ingredient preparation, mobile phase solution and blank solution for the selectivity criteria.

Precision

The repeatability (relative standard deviation, %RSD) and intermediate precision (%RSD) performed by one way balanced ANOVA are shown in (Table 4). The %RSD values for repeatability and intermediate precision did not exceed 1.7%.

Trueness

The result of trueness expressed in bias (%) and recovery (%) as can be seen in (Table 4) were assessed from the validation standards in the matrix at five concentration levels.

The trueness of the proposed method was demonstrated of *R*-isomer since the relative bias did not exceed the value of 3.57%. These values are less than ±5%, limits for active substances and in particular to ±10% for impurities in

pharmaceutical formulations, which indicated a very low bias and the absence of matrix effect.

Measurement uncertainty and uncertainty profile

The upper and lower uncertainty limit were calculated using β = 90% (proportion of accepted measurements) and γ = 95% (confidence level). The result calculated is expressed in % and presented in Table 5 shows that the uncertainty limit within the acceptance limits for assay ±10% at every concentration level studied.
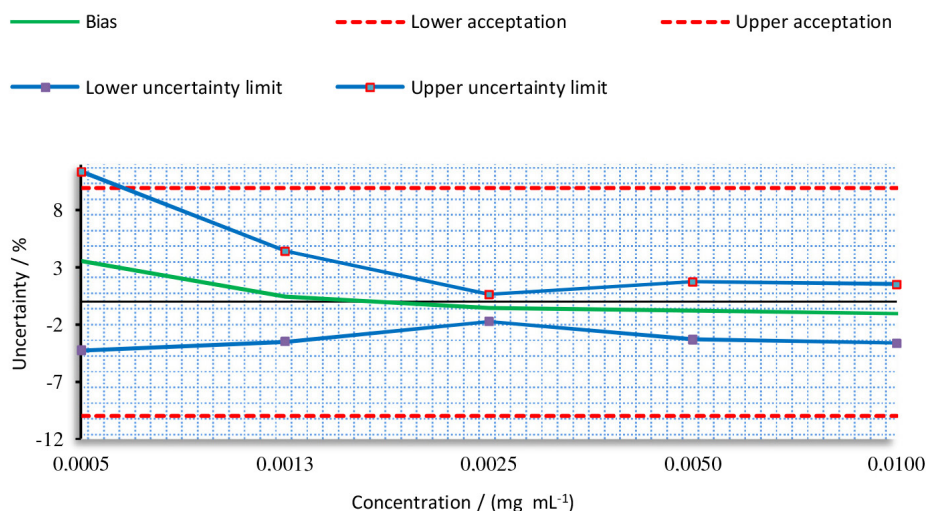
The upper and lower uncertainty limits expressed in relative value (%) are presented in Figure 4 as a function of the introduced concentrations. As can be seen from the results, the method was considered as accurate, since the uncertainty intervals are included in ±10% acceptance limits for the concentration levels tested except the lowest one. Furthermore, we conclude that the uncertainty

Table 4. Validation results for *R*-isomer (chiral impurity of Levofloxacin) using linear regression model

Concentration level / (mg mL⁻¹)	Trueness		Precision		Accuracy	
	Relative bias / %	Recovery / %	Repeatability / %	Intermediate precision / %	Lower tolerance limit / %	Upper tolerance limit / %
0.0005	3.57	103.57	1.78	1.78	−5.511	12.657
0.00125	0.46	100.46	0.69	0.69	−4.604	5.534
0.0025	−0.54	99.46	0.35	0.35	−1.928	0.845
0.005	−0.78	99.22	0.19	0.58	−5.555	4.001
0.01	−1.04	98.96	0.26	0.54	−5.352	3.265

Table 5. Point estimates of the relative expanded uncertainty and uncertainty limits obtained at each concentration level using the β-γ-content tolerance interval

Concentration level / (mg mL^{-1})	Uncertainty / (mg mL^{-1})	Expanded uncertainty / (mg mL^{-1})	Relative expanded uncertainty / %	Relative uncertainty limits / %	
				Lower	Upper
0.0005	0.00001956	0.00003913	7.82	−4.25	11.40
0.00125	0.00002479	0.00004958	3.96	−3.50	4.43
0.0025	0.00001493	0.00002986	1.19	−1.73	0.65
0.005	0.00006302	0.00012605	2.52	−3.29	1.74
0.01	0.0001289	0.00025786	2.57	−3.62	1.53

Figure 4. Uncertainty profile of *R*-isomer (chiral impurity of Levofloxacin) with β = 90% and γ = 95%. The acceptance limit is for λ = 10%.

undoubtedly exceeds the acceptance limit for the low concentration.

The validation method based on uncertainty profile approach has several advantages compared to classical approaches. First of all, this approach provides a very simple method of visual and graphical interpretation (Figure 4) that does not bother a sensitive statistical test. Furthermore, it can minimize the risk of accepting a procedure that would not be sufficiently accurate or, conversely, rejecting a procedure that would be accurate. The most important in the application of the uncertainty profile is not only to simplify the validation approach of a procedure but also the estimation of measurement uncertainty based on validation data.

Limit of quantification (LOQ)

The lower limit of quantification (LOQ) is the smallest quantity of the targeted substance in a sample that can be determined in the prescribed experimental conditions with an accuracy defined.[20,28] The LOQ was obtained by the signal/noise (S/N) ratio, the value of LOQ calculated using an experimental method was 0.000073 mg mL^{-1}.

Determination and quantification of chiral impurity in active pharmaceutical ingredient (API) of Levofloxacin

The Levofloxacin API sample was analyzed at the optimized separation conditions in order to identify and quantify the chiral impurity, and to ensure the applicability and the fitness of the optimized method. The amount of chiral impurity in the Levofloxacin API sample was calculated and the value obtained was 0.28% of the chiral impurity in the Levofloxacin API sample.

Conclusions

An analytical method for the separation and quantification of the chiral impurity of Levofloxacin was developed using an HPLC system with Chiralcel column. This method was optimised using the response surface methodology such as Box-Behnken design. Under optimised conditions, a suitable separation between Levofloxacin and its chiral impurity was observed with a resolution of 2.8 and the retention time of the chiral impurity of Levofloxacin was 13.6 min. Finally, this chiral chromatographic method was validated successfully using

uncertainty profile approach which allows us to control the risk of using the analytical method in routine and to have full information about its performance.

Supplementary Information

Supplementary data (overlay contour plot and HPLC-PDA chromatogram) are available free of charge at http://jbcs.sbq.org.br as PDF file.

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References

1. Shmid, M. G.; Gubitz, G.; *Anal. Bioanal. Chem.* **2011**, *400*, 2305.

2. Yan, H.; Row, K. H.; *Anal. Chim. Acta* **2006**, *584*, 160.

3. Shao, B.; Sun, X.; Zhang, J.; Hu, J.; Dong, H.; Yang, Y.; *J. Chromatogr. A* **2008**, *1182*, 77.

4. Tian, M.; Row, H. S.; Row, K. H.; *Monatsh. Chem.* **2010**, *141*, 285.

5. Toth, G.; Mohacsi, R.; Racz, A.; *J. Inclusion Phenom. Macrocyclic Chem.* **2013**, *77*, 291.

6. Ward, T. J.; Ward, K. D.; *Anal. Chem.* **2011**, *84*, 626.

7. Zhou, S.; Ouyang, J.; Baeyens, W. R. G.; Zhao, H.; Yang, Y.; *J. Chromatogr. A* **2006**, *1130*, 296.

8. Sun, X.; Wu, D.; Shao, B.; Zhang, J.; *Anal. Sci.* **2009**, *25*, 931.

9. Morrissey, I.; Hoshine, K.; Sato, K.; Yoshida, A.; Hayakawa, I.; Bures, M. G.; Shene, L. L.; *Antimicrob. Agents Chemother.* **1996**, *40*, 1775.

10. Saffaj, T.; Ihssane, B.; *Talanta* **2011**, *85*, 1535.

11. Saffaj, T.; Ihssane, B.; *Talanta* **2013**, *106*, 155.

12. Saffaj, T.; Ihssane, B.; Jhilal, F.; Bouchafra, H.; Laslami, S.; Sosse, S. A.; *Analyst* **2013**, *138*, 4677.

13. Saffaj, T.; Ihssane, B.; *Talanta* **2012a**, *92*, 15.

14. Ihssane, B.; Charrouf, M.; Abourriche, A.; Abboud, Y.; Bouabidi, A.; Bennamara, A.; Saffaj, T.; *Acta Chromatogr.* **2011**, *23*, 41.

15. Dejaegher, B.; Vander Hyden, Y.; *J. Pharm. Biomed. Anal.* **2011**, *56*, 141.

16. Hibbert, D. B.; *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2012**, *910*, 2.

17. Mason, R. L.; Gunst, R. F.; Hess, J. L.; *Statistical Design and Analysis of Experiments*, 2nd ed.; John Wiley & Sons: Dallas, 2003.

18. Hubert, Ph.; Nguyen-Huu, J.; Boulanger, B.; Chapuzet, E.; Chiap, P.; Cohen, V.; Compagnon, P.; Dewe, W.; Feinberg, M.; Lallier, M.; Laurentie, M.; Mercier, N.; Muzard, G.; Nivet, C.; Valat, L.; *STP Pharma Prat.* **2003**, *13*, 101.

19. Vander Heyden, Y.; Nijhuis, A.; Smeyers-Verbeke, J.; Vandeginste, B. G. M.; Massart, D. L.; *J. Pharm. Biomed. Anal.* **2001**, *24*, 723.

20. Bouabidi, A.; Rozet, E.; Fillet, M.; Ziemons, E.; Chapuzet, E.; Mertens, B.; Klinkenberg, R.; Ceccatof, A.; Talbi, M.; Streel, B.; Bouklouze, A.; Boulanger, B.; Hubert, Ph.; *J. Chromatogr. A* **2009**, *1217*, 3180.

21. Gonzalez, A. G.; Herrador, M. A.; *Talanta* **2006**, *1217*, 896.

22. Boulanger, B.; Chiap, P.; Dewe, W.; Crommen, J.; Hubert, P.; *J. Pharm. Biomed. Anal.* **2003**, *32*, 753.

23. Feinberg, M.; *Labo-Stat: Guide de Validation des Méthodes d'Analyse*, Lavoisier, TEC &DOC: Paris, France, 2009.

24. Feinberg, M.; *Validation des Méthodes d'Analyse Quantitatives au Moyen du Profil d'Exactitude*. Techniques de l'ingénieur, T.I & Weka: France, 2010.

25. Zufia, L.; Aldaz, A.; Ibanez, N.; Viteri, C.; *J. Pharm. Biomed. Anal.* **2009**, *49*, 547.

26. Streel, B.; Cahay, B.; Klinkenberg, R.; *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2009**, *877*, 2290.

27. Barwick, V. J.; Ellison, L. R.; *VAMProject 3.2.1, Development and Harmonization of Measurement Uncertainty Principles. Part D: Protocol Uncertainty for Evaluation from Validation Data*. Report No.:LGC/VAM/1998/088. Accelrys Corporate Headquarters: San Diego, USA, 2000.

28. Hubert, P.; Nguyen-Huu, J. J.; Boulanger, B.; Chapuzet, E.; Chiap, P.; Cohen, N.; Compagnon, P. A.; Dewé, W.; Feinberg, M.; Lallier, M.; *J. Pharm. Biomed. Anal.* **2007**, *45*, 70.

29. Ihssane, B.; Sbai, M.; El hadrami, El.; Achour, S.; Saffaj, T.; *Int. J. Pharm. Pharm. Sci.* **2014**, *6*, 413.

30. Tan, A.; Saffaj, T.; Musuku, A.; Awaiye, K.; Ihssane, B.; Jhilal, F.; Alaoui, S. S.; Trabelsi, F.; *J. Chromatogr. B: Biomed. Sci. Appl.* **2015**, *983*, 68.

31. Mee, R.; *Technometrics* **1984**, *26*, 251.

32. Satterthwaite, F. E.; *Biom. Bull.* **1946**, 110.

33. Wu, L.; Yick , K. L.; Ng, S. P.; Yip, J.; *Expert Sys. Appl.* **2012**, *39*, 8059.