Journal of Pharmaceutical and Biomedical Analysis 54 (2011) 694–700

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Optimisation and validation of a fast HPLC method for the quantification of sulindac and its related impurities

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ARTICLE INFO

Article history:
Received 23 July 2010
Received in revised form 19 October 2010
Accepted 27 October 2010
Available online 3 November 2010

Keywords:
HPLC
Sulindac
Validation
Design space
Sub-2micron column

ABSTRACT

The European Pharmacopoeia describes a liquid chromatography (LC) method for the quantification of sulindac, using a quaternary mobile phase including chloroform and with a rather long run time. In the present study, a new method using a short sub-2 μm column, which can be used on a classical HPLC system, was developed. The new LC conditions (without chloroform) were optimised by means of a new methodology based on design of experiments in order to obtain an optimal separation. Four factors were studied: the duration of the initial isocratic step, the percentage of organic modifier at the beginning of the gradient, the percentage of organic modifier at the end of the gradient and the gradient time. The optimal condition allows the separation of sulindac and of its 3 related impurities in 6 min instead of 18 min. Finally, the method was successfully validated using an accuracy profile approach in order to demonstrate its ability to accurately quantify these compounds.

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1. Introduction

Sulindac is a non-steroidal anti-inflammatory drug with a poor pharmacological activity. It can be metabolised by reversible reduction into a sulphide metabolite, with a high pharmacological activity, or by irreversible oxidation into a sulphone metabolite, which has no pharmacological activity [1]. E-sulindac is an impurity resulting from the synthesis of sulindac. Sulindac is used for the treatment of acute or chronic inflammatory conditions. High-performance liquid chromatography (HPLC) is the most commonly used technique for the analysis of sulindac and its related impurities [2–6]. The European Pharmacopoeia (Eur. Ph.) [7] describes a normal phase HPLC method for this purpose. However, this method requires a quaternary mobile phase containing chloroform and a rather long analysis time. In pharmaceutical, biomedical and food analysis, the recent trend has been to develop fast analysis methods (fast-HPLC) [8–11].

Over the last decade, different strategies have been developed to reduce the analysis time while maintaining efficiency or to improve efficiency with a similar analysis time [12,13]. One of these strategies, ultra-performance liquid chromatography (UHPLC) uses a column packed with sub-2 μm particles. However, such small particles generate high back pressure and necessitate the use of appropriate and quite expensive equipment able to withstand such an ultra high pressure (up to 1200 bar). In order to reduce back pressure, some column manufacturers put forward the use of short sub-2 μm columns with a higher internal diameter. The advantage of this type of column is that it can be used with conventional HPLC equipment (up to 400 bar) [14]. In this study, a Platinum C18 Rocket column (53 × 7 mm i.d., 1.5 μm particle size) was used. This column provides a shorter analysis time, a lower solvent, faster equilibration, accurate quantitation and high efficiency [15,16]. Some recent papers report methods based on this brand of column for the determination of pharmaceutical substances in plant tissues [17–19], for the monitoring of amiodarone [20], or for the separation of microcystins and nodularins on narrow-bore [21]. In this study, a new fast and easy HPLC method was developed without the use of chloroform for the separation and determination of sulindac and its related impurities. This method was optimised in terms of separation by using design of experiments (DoE) methodology [22–24] and the design space (DS) concept [25,26]. Finally, the method was validated using the accuracy profile approach based on a β-expectation tolerance interval [27–32].

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doi:10.1016/j.jpba.2010.10.022

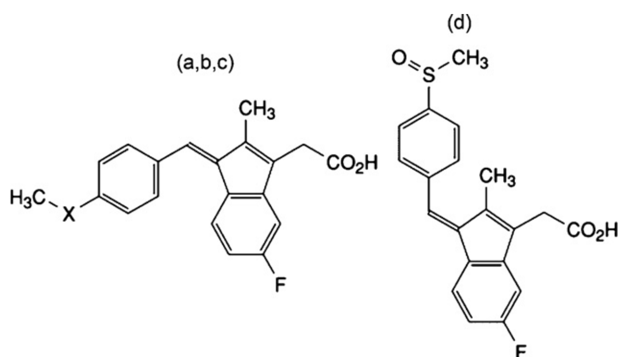

Fig. 1. Chemical structures of sulindac and its impurities. (a) Sulindac (X = SO), (b) sulindac sulphone (X = SO_2), (c) sulindac sulphide (X = S), (d) E-sulindac.

2. Materials and methods

2.1. Chemicals and solvents

Acetonitrile (LiChrosolv, 99.9%), acetic acid (99.8%) and ethyl acetate for analysis (99.5%) were purchased from Merck (Darmstadt, Germany). Absolute ethanol (99.99%) was purchased from Fisher Scientific (Loughborough, UK) and chloroform Chromasolv (99.8%) from Sigma–Aldrich (Buchs, Switzerland). Sulindac was supplied by EP (Strasbourg, France). Sulindac sulphone and sulindac sulphide were purchased from Sigma (Steinheim, Germany). E-sulindac was supplied by the United States Pharmacopeia (Rockville, USA). The chemical structures of sulindac and its related impurities chemical structures are shown in Fig. 1. Water was purified using a Millipore system (18.2 MΩ/cm resistivity, Milli-Q) and filtered through a 0.22 μm Millipore Millipak®-40 disposable filter unit (Millipore Corporation, USA). Phosphate buffer solution (pH 7.4; 50 mM) was prepared by dissolving 27.2 g of potassium dihydrogen phosphate (Merck), 6.3 g of sodium hydroxide (VWR, Leuven, Belgium) and 0.64 g of sodium azide (Merck) in 2573.6 mL deionised water. The pH value was adjusted to 7.4 with 0.1 M sodium hydroxide solution. Phosphate buffer solution (pH 2.0; 10 mM) was prepared by dissolving 4 mL phosphoric acid (Aldrich) and 12.12 g potassium dihydrogen phosphate (Merck) in 2000.0 mL deionised water. The pH value was then adjusted to 2.0 with a 1.2 M hydrochloric acid solution.

2.2. Apparatus

Analyses were performed on an Agilent technologies HPLC 1100 series system (Agilent Technologies, Santa Clara, CA, USA) equipped with a solvent delivery binary pump G1312A, an on-line degasser G1379A, a thermostatised autosampler G1328A, a column oven G1316A and a diode-array detector G1316A. Chemstation® (Rev.B.01.03[204]) was used to control the whole chromatographic system and to acquire, process and store all the data obtained. A Mettler Toledo (Schwerzenbach, Switzerland) MX5 microbalance was used to weigh all the compounds (precision: 1 μg). A Seven Easy Mettler Toledo pH meter was used to adjust the pH value.

2.3. Chromatographic conditions

2.3.1. Reference method [7]

The reference method used is the one described for a related substance of sulindac in Eur. Ph. 6.7. This reference method is in the normal phase mode. Chromatographic analyses were performed on an Alltima Silica column (250 × 4.6 mm i.d., 10 μm particle size), which was kept at 20 °C. The mobile phase was prepared by mixing acetic acid, ethanol, ethyl acetate and chloroform (1:4:100:400 (v/v/v/v)) and was degassed before use. The HPLC system was operated in isocratic mode at a flow rate of 2.0 mL/min and the injection volume was 20 μL. UV detection was performed at 280 nm.

2.3.2. Optimised method

Sulindac is a weak acid and has a pK_a of 4.7. Related impurities have a pK_a near to 4.7. Then, it was decided to work with a mobile phase composed of acetonitrile and a phosphate buffer solution (pH 2.0; 10 mM). At the selected mobile phase pH, the compounds are almost totally non-ionised. Thus, to have good separation, a C18 silica packed column was chosen. The chromatographic analyses were performed on a Platinum C18 Rocket column (53 × 7 mm i.d., 1.5 μm particle size), which was kept at 35 °C. Despite the high internal diameter of the column, in order to avoid being concerned by too high pressures, acetonitrile was selected as an organic modifier as it has a lower viscosity than methanol. The mobile phase proportion and the gradient shape were optimised by means of DoE methodology in order to find the design space (DS) (see Section 3.4). The injection volume was 100 μL. UV detection was performed at 340 nm. The HPLC system was operated with a gradient mode at a flow rate of 3.0 mL/min. Following the gradient method transfer rules [9] with as a starting column geometry, a conventional C18 150 × 4.6 mm i.d. (5 μm) operated at 1 mL/min, the Platinum C18 Rocket column should be used with a 7.7 mL/min flow rate. Nevertheless, at this flow rate, the back pressure is higher than the maximal operating back pressure on an Agilent technologies HPLC 1100 series system (i.e. >350–400 bar). Therefore, to maintain a backpressure lower than 350 bar and not too much sacrificing performance, the flow rate was adjusted to the above mentioned value of 3.0 mL/min. Thanks to the high column internal diameter, the back pressure obtained at this flow rate was only about 280 bar.

2.4. Standard solutions

A stock solution of sulindac was prepared by dissolving 50.0 mg of sulindac in 50.0 mL of methanol (1 mg/mL). A stock solution of E-sulindac, sulindac sulphide and sulindac sulphone was prepared by dissolving 2.0 mg of each compound in 20.0 mL of methanol. The calibration and validation standards [27–29] were prepared by mixing and diluting the stock solutions with phosphate buffer solution (pH 7.4; 50 mM) to reach the concentration levels: 100/10; 100/5; 100/1; 100/0.5; 50/0.25; 25/0.125; 1/0.005; 0.5/0.0025 μg/mL (sulindac concentration/concentrations of related impurities, respectively).

3. Theory/calculation

3.1. Computations

The experimental design results were processed using the software R version 2.7.2. The validation data were processed with the software e-noval version 2.0e (Arlenda s.a., Liège, Belgium).

3.2. Experimental design

Four HPLC factors were investigated using DoE methodology through a design matrix. All of the factors were quantitative:

- The percentage of acetonitrile at the beginning of the gradient (ACNlower),
- The percentage of acetonitrile at the end of the gradient (ACNupper),
- The gradient time (Gradient time),
- The duration of the initial isocratic step (Plateinit).

Table 1
Description of the levels of four factors involved in the experimental design.

	Plateinit (min)	ACNlower (%)	ACNupper (%)	Gradient time (min)
Levels	0-1	15-30-55	55-60-65	1-3-5
Central point	0-0.5-1	30	60	3
Maximum effect	Linear	Quadratic	Quadratic	Quadratic

To assess the reproducibility of runs, three central points were repeated independently twice for each "Plateinit" level (see Table 1). In addition, quadratic effects were assumed for each factor except for "Plateinit" which was considered as linear. In order not to miss an effect or interaction which would not be assessed or modelled by the chromatographic theory, no a priori knowledge about the empiric compound behaviour was added in the multiple linear models. A full factorial design was chosen to explore the experimental domain. 58 experiments (3^3 2 + 4) were carried out in order to explore the experimental domain. It is important to bear in mind that these experiments were used to models the chromatographic behaviour of each peak and concurrently evaluate the method robustness. In practice, experiments were sufficiently rapid and easily carried out to overcome this high number of experiment. Levels of each factor are summarized in Table 1.

3.3. Statistical models

In the resulting chromatograms, four peaks were detected and indexed at their beginnings, apexes (retention time) and ends as proposed by Lebrun et al. [26]. The logarithms of the retention factors ($\log(k)$) were selected as responses of the multi-linear model following Eq. (1).

$$\begin{aligned}\log(k) \; &= \beta_0 + \beta_1\,\text{Plateinit} + \beta_2\text{ACNlower} + \beta_3\,\text{ACNlower}^2 \\ &+ \beta_4\,\text{ACNupper} + \beta_5\,\text{ACNupper}^2 + \beta_6\,\text{Gradient} \\ &+ \beta_7\,\text{Gradient}^2 + \beta_8\,\text{ACNlower Plateinit} \\ &\times \beta_9\,\text{ACNupper Plateinit} + \beta_{10}\,\text{Gradient Plateinit} \\ &+ \beta_{11}\,\text{ACNlower Plateinit} + \beta_{12}\,\text{ACNlower Gradient} \\ &+ \beta_{13}\,\text{ACNupper Gradient} + \varepsilon,\end{aligned} \quad (1)$$

where $\beta_0, \ldots, \beta_{13}$ are the parameters to estimate and ε is the predictive error, for instance using the classical least squares regression.

3.4. Optimisation – design space

The separation of the critical pair (i.e. the two most proximate peaks) was optimised using the methodology presented in the publications of Lebrun et al. and Dewé et al. [26,33]. The design space (DS) is the zone of the experimental domain where the quality of the chromatogram is high. The separation criterion is defined as the difference between the beginning of a peak and the end of the preceding peak. This criterion was calculated from the predicted retention times. The analysis of the model's predictive error was also carried out to find the DS, i.e. the set of operating conditions that gives a high probability of obtaining a separation criterion higher than a given threshold. Mathematically, the DS is defined as in Eq. (2),

$$\mathrm{DS} = \left\{ \mathbf{x}_0 \in \chi | E_{\hat{\theta}}[P(\min(S) > \lambda)|\hat{\theta}] \geq \pi \right\} \quad (2)$$

where $\mathbf{x}_0$ is the set of factor conditions belonging to the experimental domain χ, for which the expected probability of having a minimal separation ($\min(S)$) higher than λ, is higher than π, given the uncertainty of the estimation of model parameters $\theta = (\beta_0, \ldots, \beta_{13}, \varepsilon)$.

The objective of this study was to determine the optimal chromatographic conditions allowing us to obtain a separation criterion of at least $\lambda = 0$ min (i.e. baseline resolved peaks) with a probability of at least 90% ($\pi = 0.9$). Monte-Carlo simulations were then performed to propagate a predictive error from responses to the separation criterion. This step was carried out to estimate the error affecting the criterion in order to compute the probability of reaching the separation time of 0 min [33].

4. Results and discussion

4.1. Reference method

As can be seen in Fig. 2, the reference method enabled the separation of all the compounds within 18 min and was completed within 25 min. Under these conditions, the separation of all compounds was acceptable. The Eur. Ph. method uses chloroform, which is now considered as a toxic organic solvent. Furthermore, the relatively long analysis time involved in that method, in combination with a high flow rate of 2 mL/min, implies a large consumption of chloroform. Consequently, it was of particular interest for us to develop a new method avoiding the use of this kind of solvent and presenting a shorter analysis time.

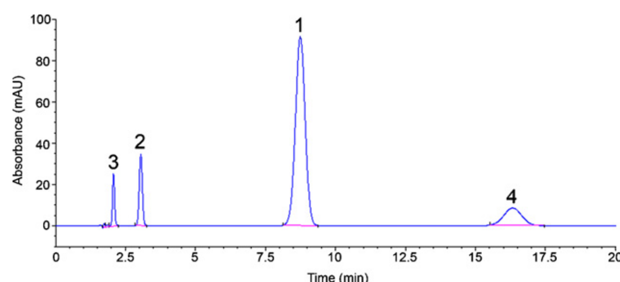

Fig. 2. Reference method chromatogram (European Pharmacopoeia 6.7 (1: sulphide, 2: sulphone, 3: sulindac, 4: E-sulindac)).

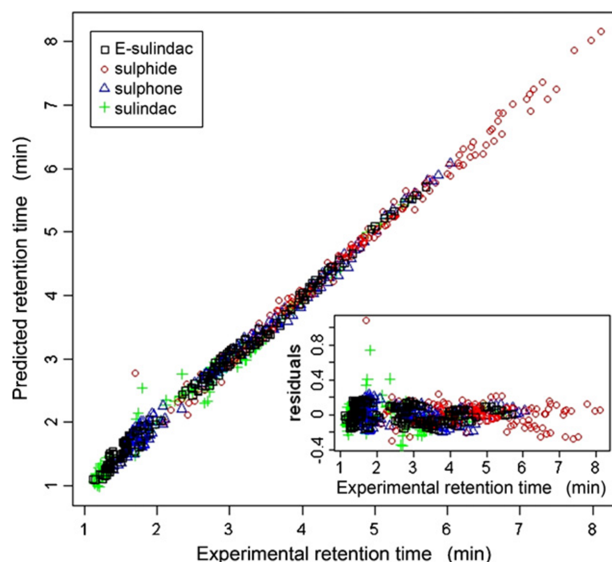

Fig. 3. Experimental retention times versus predicted ones. Residuals are depicted at the bottom right corner.

4.2. Optimised method

Taking into account the fact that the aim of this study was to develop a short run time method, “Plateinit” was limited to 1 min, gradient time to 5 min and after some preliminary screening experiments, “ACNlower” was set from 15 to 55% and “ACNupper” from 55 to 65%. Indeed, a percentage of “ACNlower” less than 5% is not recommended for the integrity of the column while a percentage higher than 55% would not be able to separate satisfactorily the different compounds. Meanwhile, a percentage of “ACNupper” higher than 65% increased drastically the slope of the gradient. A baseline drift could be prejudicial to the quality of the quantitative results. Fig. 3 illustrates the quality of the fit of the observed retention times versus the predicted retention times using the statistical models previously described. Most of the residuals were mainly located within the interval [−0.2 min, 0.2 min]. However, the overall quality of fit was considered as good as the adjusted R^2 values for each model were higher than 0.95. Fig. 4 shows the probability surfaces in different directions of the space around the optimal solution (for each graph, two factors were fixed at their optimal values). As we can see, the best probability surface was obtained when the duration of the initial isocratic plate was around 1 min. The probability of separating the critical pair is higher when the gradient time was around 3.6 min: when this time increases, the probability of separation decreases. We found a good probability of separation when the percentage of organic modifier at the

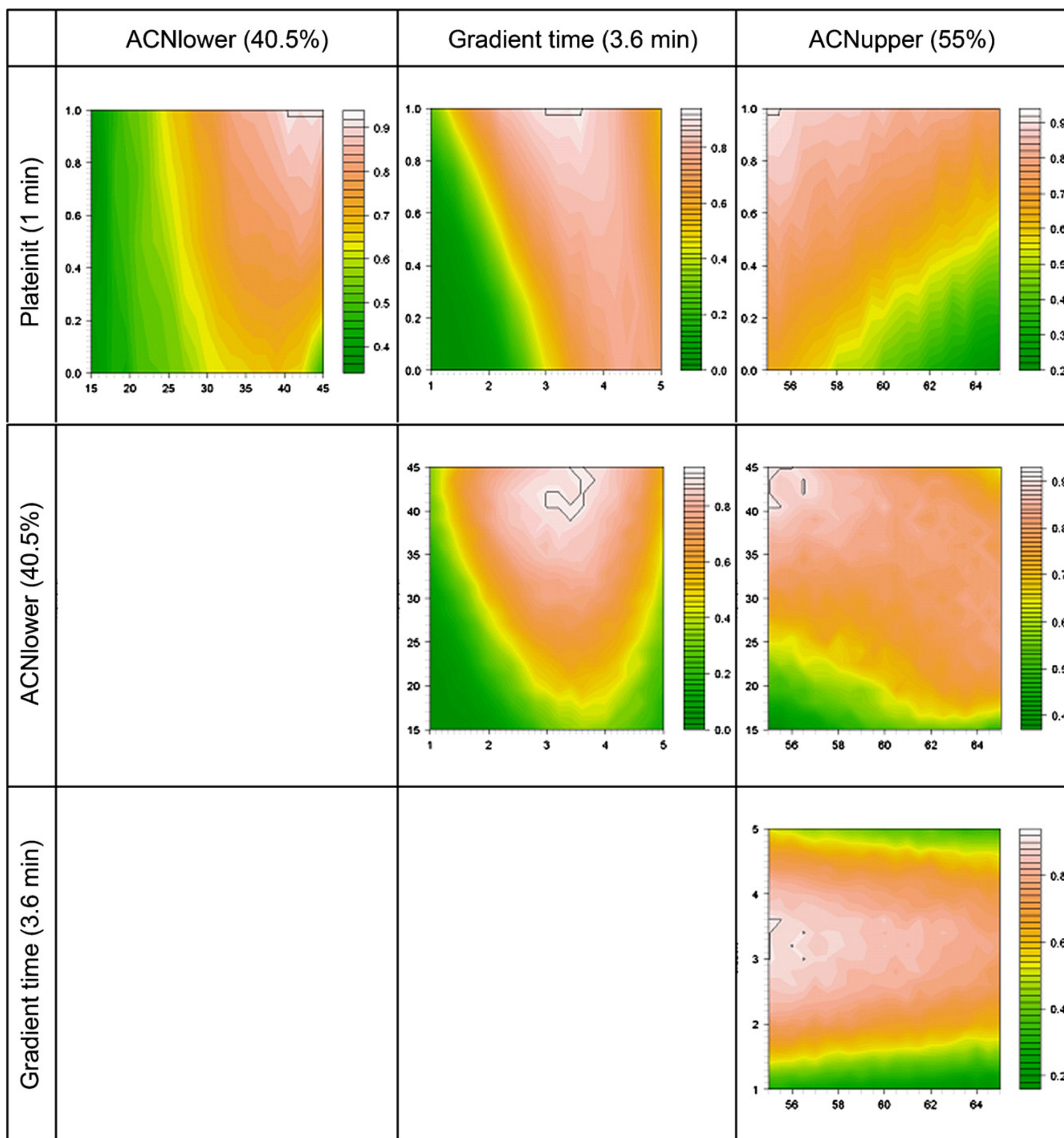

Fig. 4. Surface of probability to reach $S > 0$. The design space is surrounded by black lines for an expected probability to have well-separated peaks is 0.9. Factors optimal values are placed between parentheses.

beginning of the gradient was between 40 and 45% and when the percentage of organic modifier at the end of the gradient was 55%. The optimal values allowing the achievement of the higher probability (P(separation > 0) > 0.9) ensuring a separation of at least 0 min with baseline-resolved peaks were 1 min for the Plateinit, 40.5% for the ACNlower, 55% for the ACNupper and the gradient time was 3.6 min.

The developed HPLC method for the quantification of sulindac and its related impurities divided the run time of analyses by three compared to the reference method. Fig. 5a and b shows the optimal predicted and experimental chromatograms. As can be seen, the predicted retention times were found to be very close to the experimental values and an acceptable separation was obtained within an analysis time of 6 min.

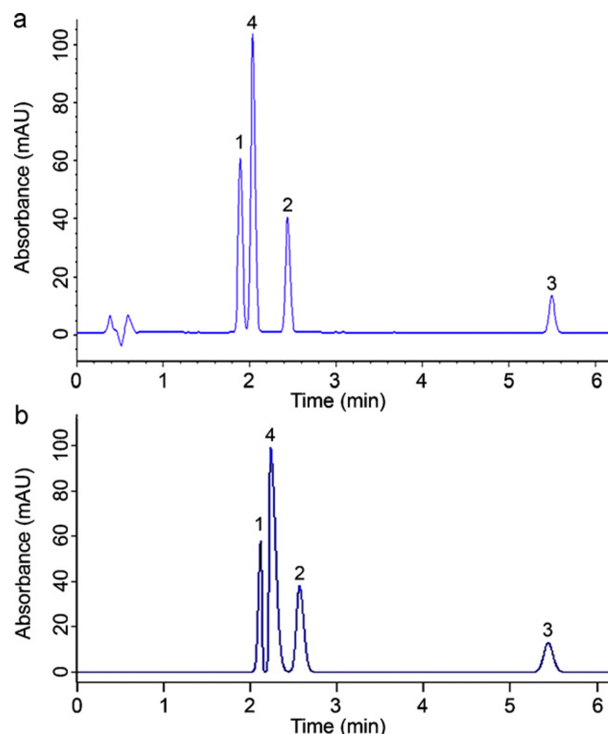

Fig. 5. (a) Experimental chromatogram recorded at optimal solution. (b) Predicted chromatogram at optimal condition (1: sulindac, 2: sulphide, 3: sulphone, 4: E-sulindac).

4.3. Validation method

The aim of this validation was to establish that the analytical method was suitable for the quantitative determination of the 4 compounds described above. In other words, it was to demonstrate the method's ability to quantify them. Several widely recognised validation criteria were evaluated (selectivity, trueness, precision, accuracy, and limits of quantification and detection) in accordance with ICH Q2(R1) guidelines [31]. An original approach using accuracy profiles based on tolerance intervals was applied to evaluate the reliability of the results. The tolerance interval used was a "β-expectation tolerance interval" defining an interval in which it is expected that each future result will fall with a defined probability (β). It is therefore a predictive methodology. This tolerance interval is computed for each validation standard concentration level, using their estimated intermediate precision standard deviation and bias. By joining together the upper tolerance limits on the one hand and the lower tolerance limits on the other hand, the method defines an accuracy profile. As long as this profile stays within the acceptance limits set according to the needs of the final user or to regulatory expectations the method can be considered as valid. Indeed, it guarantees that each future result will be included in the a priori set acceptance limits with at least a probability of β (e.g. 0.95%).

4.3.1. Response function

The response function represents the relationship between the detector response and the concentration of the analyte in the sample. In order to determine the most appropriate calibration curve, different models were evaluated. Due to the wide concentrations range, the calibration curve for sulindac was built with 5 calibration standards, from 0.5 μg/mL to 100.0 μg/mL. Independent validation standards were also prepared (5 validation standards from 0.5 μg/mL to 100.0 μg/mL). For the related impurities (E-sulindac, sulindac sulphone and sulindac sulphide), the concentrations range of the calibration standards was reduced. Only 4 calibration standards from 10 μg/mL to 0.125 μg/mL were released. Each calibration and validation standard was analysed in triplicate and four consecutive series were performed in order to evaluate the intermediate precision. The concentration of the validation standards was then back-calculated to determine the mean relative bias as well as the standard deviation for repeatability and intermediate precision, in accordance with the work of Rozet et al. [34]. On this basis, different accuracy profiles were then plotted to select the most appropriate calibration model for the analytical method. The acceptance limits were set at ±5% for sulindac and ±15% for its related substances and the upper and lower β-expectation tolerance interval were set at 95%. The best response function was achieved with a weighted ($1/X$) quadratic regression transformation for sulindac, with linear regression after logarithm transformation for E-sulindac and sulphone and with a linear regression through 0 fitted with the higher level of concentration only for sulphide.

4.3.2. Trueness

Trueness is defined as the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value [31]. From the results obtained and considering the appropriate model for each compound, the concentration of the validation standards was back-calculated to determine trueness expressed in terms of relative bias (%). As can be seen in Table 2, the relative biases never exceeded 1% for sulindac or 7% for the impurities.

4.3.3. Precision

Precision is defined as the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions [31]. Precision was computed here using the relative standard deviations (RSDs) for repeatability and intermediate precision at each concentration level of the validation standards. The results are indexed in Table 2. It can be seen that RSD values never exceeded 2 or 4% for repeatability or intermediate precision, respectively, illustrating the good precision of the analytical method.

4.3.4. Accuracy

Following ICH Q2(R1) guidelines [31], accuracy is defined as the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Upper and lower β-expectation tolerance intervals (%) were calculated and did not exceed the acceptance limits for each concentration level, as shown in Fig. 6. These observations proved that the HPLC method used in this study was accurate over the studied concentration range. In fact, β-expectation tolerance interval based accuracy profiles allow us to guarantee that a high

Table 2
Validation criterion for sulindac, E-sulindac, sulphone and sulphide.

Validation criteria	Sulindac	E-sulindac	Sulphide	Sulphone
Response function	Weighted (1/X) quadratic regression	Linear regression after logarithm transformation	Linear regression through 0	Linear regression after logarithm transformation
Trueness (concentration (μg/ml)/relative bias (%))				
Level 1	101.40/−0.01	10.25/3.39	9.74/−0.05	10.13/2.37
Level 2	50.68/0.43	5.12/−1.71	4.87/−0.45	5.06/−3.36
Level 3	25.32/0.04	1.025/−2.72	0.97/3.46	1.01/0.08
Level 4	1.01/0.01	0.13/−0.29	0.12/6.63	0.13/−0.11
Level 5	0.51/0.78	–	–	–
Precision (repeatability (RSD%)/intermediate precision (RSD%))				
Level 1	0.53/1.38	0.45/1.76	0.43/1.28	0.57/1.54
Level 2	0.53/0.60	0.58/0.98	0.45/0.96	0.57/1.08
Level 3	0.60/0.70	0.89/1.37	0.87/1.93	0.83/1.77
Level 4	0.67/0.67	1.61/3.93	0.71/2.71	0.92/2.70
Level 5	0.94/1.51	–	–	–
Accuracy (lower β-EL (%)/upper β-EL (%))				
Level 1	−4.22/4.19	−2.57/9.35	−4.23/4.14	−2.58/7.31
Level 2	−0.99/1.85	−4.46/1.03	−3.37/2.47	−6.53/0.19
Level 3	−1.65/1.74	−6.47/1.02	−2.47/9.40	−5.27/5.44
Level 4	−1.53/−1.55	−12.63/12.05	−2.45/15.77	−8.90/8.69
Level 5	−3.39/4.96	–	–	–
Linearity				
Slope	0.9997	1.0330	0.9969	1.020
Intercept	0.0422	−0.0792	0.0159	−0.0659
R^2	0.9996	0.9989	0.9997	0.9988

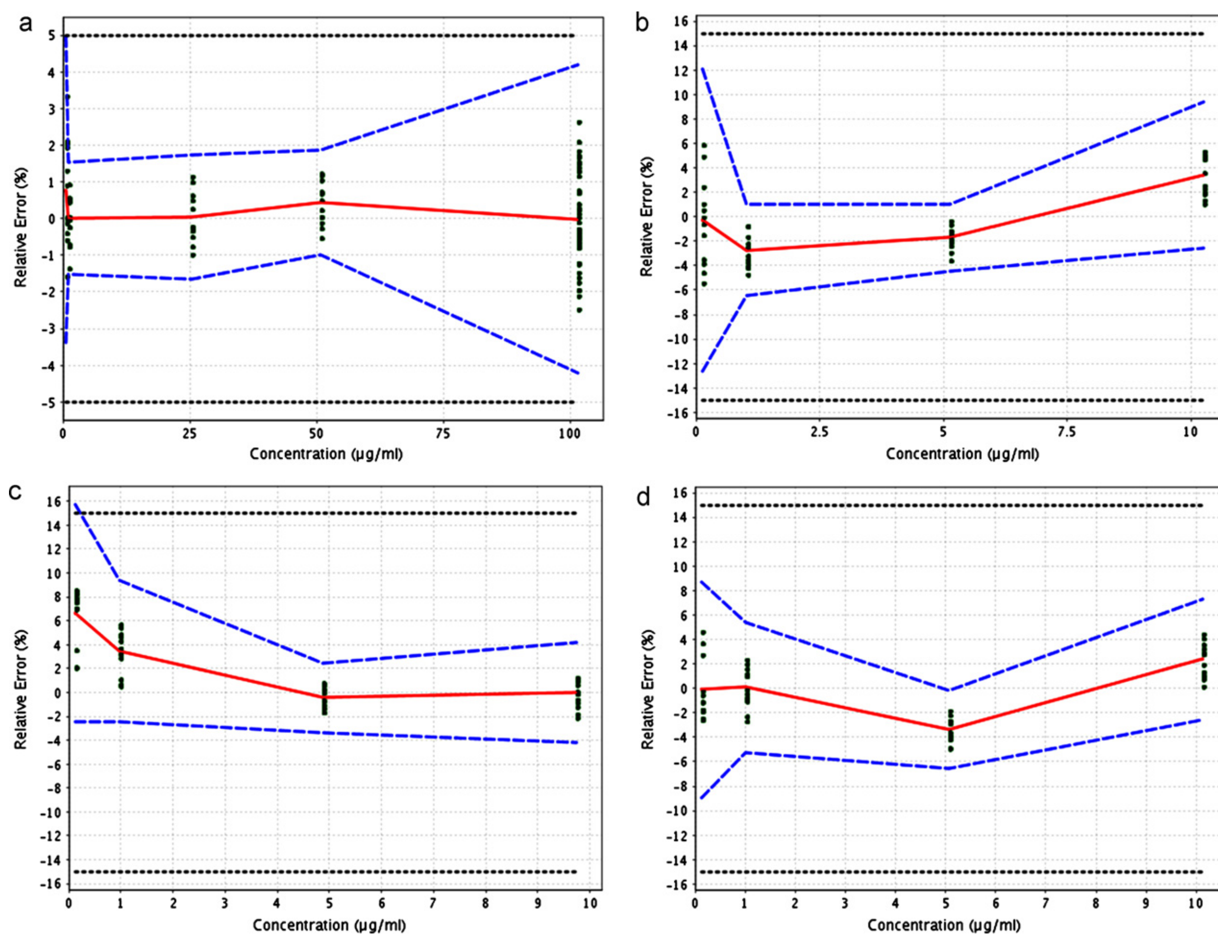

Fig. 6. Accuracy profiles of (a) sulindac, (b) E-sulindac, (c) sulphide and (d) sulphone. Relative bias (—), ±5% acceptance limits (- - -), 95% (sulindac) or 85% (related impurities) β-expectation tolerance limits (– – –), and relative back-calculated concentrations (●).

percentage (e.g. 95%) of future results will lie within the acceptance limits [31].

4.3.5. LOQs and LODs

The limits of quantification (LOQs) were obtained by calculating the smallest and highest concentration level beyond which accuracy limits or β-expectation limits go outside the acceptance limits. The lower and upper limits of quantification were evaluated from the corresponding accuracy profile at 0.5063 and 101.4 μg/mL for sulindac, at 0.1281 and 10.25 μg/mL for E-sulindac, at 0.2244 and 9.738 μg/mL for sulphide and at 0.1266 and 10.13 μg/mL for sulphone. The limits of detection (LODs) were computed at 0.1380 μg/mL for sulindac, at 0.0388 μg/mL for E-sulindac, at 0.0680 μg/mL for sulphide and at 0.0383 μg/mL for sulphone.

5. Conclusions

An analytical method for the quantification of sulindac and its related impurities was developed using a short column with sub-2 μm particles on a classical HPLC system. This method was optimised using DoE methodology and the DS concept. Under optimised conditions, the analysis time was considerably reduced (by about 3-fold). The solvent consumption was reduced by about 2-fold and the equilibration was reduced by about 1.5-fold. Furthermore, we did not use chloroform unlike in the Eur. Ph. reference method. Finally, this particular method was validated successfully using an accuracy profiles approach for sulindac and its related substances. Several criteria were successfully tested (trueness, precision, accuracy, and limits of quantification and detection).

Acknowledgements

We would like to thank Stéphane Counerotte and Laurence Collard for their technical help. The author would also like to thank the Walloon Region of Belgium for the FIRST-DEI convention funds N°516130, 516138 and for the Waléo 2 convention funds N° 516266.

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