Quality by Design (QbD) Approach to Develop and Validate a HPLC Method for Piroxicam from Serum

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Piroxicam belongs to the non-steroidal antiinflammatory class, it is utilized as analgesic, anti-inflammatory and antipyretic. A simple and accurate HPLC method was optimized and validated in the QbD approach, for the determination of piroxicam in serum. The optimization of HPLC analysis was performed using different columns, mobile phases and flows. The HPLC method was appropriately validated for linearity, limit of detection, limit of quantification, precision and accuracy in accordance with the ICH guidelines. The optimized and validated method proved to be simple, precise, and accurate, and can be successfully applied for the determination of piroxicam in serum.

Keywords: piroxicam, QbD, HPLC, serum, validation

Piroxicam, [4-hydroxy -2-methyl-N -2 – (pyridyl) -2H - 1, 2 -benzothiazine-3- carboxamide - 1, 1 - dioxide], is a non-steroidal antiinflammatory drug (NSAID) belonging to a oxicam class. It is utilized as analgesic, anti-inflammatory and antipyretic in the treatment of rheumatoid arthritis, osteo-arthritis, ankylosing spondylitis, gout, musculoskeletal disorders, post partum pain and sport injuries. Its main action is the reversible inhibition of the cyclooxigenase causing the inhibition of prostaglandins synthesis [1]. It shows also chemopreventive and chemosuppresive activity in different cancerous cell lines [2-4]. At high concentrations gastrointestinal and hepatologic side effects such as liver failure, gastroduodenal erosions, or even bleeding ulcers have been described [5, 6]. Therefore, the analysis of piroxicam in the pharmaceutical formulations and in the biological fluids is necessary for obtaining optimum therapeutic concentration and for assure the quality of products. Several analytical methods have been described for the quantitative determination of piroxicam in pharmaceuticals and in the biological fluides. Of these we mention spectrophotometric methods [7-14], spectrofluorimetric methods [15, 16] and Infrared spectrometry [17]. Thin-layer chromatographic (TLC) separation methods and high - performance liquid chromatography (HPLC) has been used for quantitative analysis of piroxicam [18-25]. Also, electrochemical technique were applied for separation and quantitative determination of piroxicam [26-29].

Attempts, therefore, were made to develop and validate a rapid, sensitive, robust and economical bioanalytical HPLC method for estimating piroxicam in plasma using QbD-oriented systematic analytical approach. The studies were carried out in three phases, that is, initial factor screening for identifying the CMVs, optimization of chromatographic conditions and method validation.

Quality by Design (QbD) is a concept first outlined by Joseph M. Juran, most notably in Juran on Quality by Design [30], where he presents a new and exhaustively comprehensive approach to planning, setting, and reaching quality. Since first initiated by the FDA [31], the QbD has become an important concept for the pharmaceutical industry. This is further defined in the International Conference on Harmonisation (ICH) guidance on pharmaceutical development as *a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management* [32, 33].

In the QbD approach, the quality is not assured by testing and inspections but the quality is built into product & process by design and based on scientific understanding, it includes knowledge rich submission which shows product knowledge & process understanding. *Analytical Quality by Design (AQbD) Method Validation* approach is the validation of analytical method over a range of different batches. It uses both *Design of Experiments* (MOD-DoE), *Method Operable Design Region* (MODR) knowledge for designing method validation. The approach provides the required ICH validation elements as well as information on interactions, measurement uncertainty, control strategy, and continuous improvement. This approach requires fewer resources than the traditional validation approach without compromising quality. ICH Q11 [34] has explained the QbD approach for API synthetic process development but there is no specific discussion on AQbD. However, it is recommended to implement QbD approach in analytical method development termed as AQbD. These two scientific approaches (QbD and AQbD) can be progressed in equal time [35-38].

Based on the principles of sound science and quality risk management, the QbD approach enables enhanced understanding of the Critical Method Variables (CMVs) influencing the Critical Analytical Attributes (CAAs) and the method performance. Not only does design of experiments help in identifying the 'vital few' method variables critically influencing the method performance, but it also assists in optimizing them, while expending minimal resources of time, effort and cost [39].

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Critical Quality Attributes (CQA) and Initial Risk Assessment

*Critical Quality Attributes (*CQA) for analytical methods includes method attributes and method parameters. The CQAs for a HPLC method are diluents, column selection, mobile phase composition (organic modifier, buffer, *p*H), and elution method.

Risk Assessment is a science-based process used in quality risk management and it can identify the material attributes and method parameters. Risk Assessment can be performed from initial stage of method development to continuous method monitoring. AQbD approach involves the risk identification at early stages of development followed by appropriate mitigation plans with control strategies that will be established. In general, Ishikawa fishbone diagram can be used for risk identification and \ assessment. See figure 1 that shows fishbone risk identification approach for typical analytical test procedure.

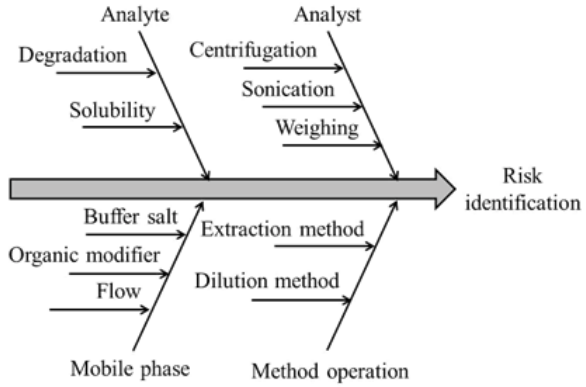

Fig. 1. Fishbone for Risk identification

Design of Experiments (DoE) - Method Optimization and Development

After the potential and critical analytical method variables are defined with initial risk assessment, the DoE can be performed to confirm and refine critical method variables based on statistical significance. As per ICH Q8 guidance process robustness is defined as *Ability of a process to tolerate variability of materials and changes of the process and equipment without negative impact on quality* [34]. Process understanding will provide the sufficient knowledge for establishing robustness parameters by evaluating different operating conditions, difference scales, and different equipments [37].

Experimental part

Reagents, standards and test solutions

Acetonitrile and methanol of chromatographic purity and sodium acetate of analytical purity were purchased from Merck's Chemical Co., Darmstadt, Germany. HPLC grade water was obtained from Milli-Q water purification system (Millipore, Milford, USA).

The standard solution was prepared by dissolving piroxicam (reference substance) in methanol and diluting the obtained solution with methanol at a final concentration of 20 mg/mL. From this, were prepared solutions in the concentrations range of 0.5 to 10 mg/mL by diluting with methanol.

Working solutions with concentrations ranging from 0.5 to 10 μg/mL were made by appropriate serial dilution with methanol, 1 mL from these solutions were mixed with 1 mL serum, vortexed vigorously for 10 min on a vortex mixer. Test solutions were obtained by mixing 1 mL sample serum with 1 mL methanol. The working solutions or the samples were then centrifuged at 4000 rpm, for 30 min to assure phase separation. A volume of 20 μL from supernatant was analyzed by HPLC in the described conditions.

Equipment and chromatographic conditions

An Agilent 1100 system consisting of a high-pressure pump with an on-line degasser, manual injector, a column oven, and a diode array detector (for determining spectral peak purity) was used. For sample preparation a Kern 770 analytical balance, an ultrasonic bath and a Cencom II centrifuge were used. The column used were Eclipse XDB C18 (150 mm x 4.6 mm, 5 μm) and Zorbax SB C18 (150 mm x 3 mm, 3.5 μm) types, at constant temperature of 25°C. Separation was performed in isocrat mode using a mobile phase consisting of a mixture of water or acetate buffer/acetonitrile/methanol in different proportions and different flow rate. The injected volume was 20 μL. Detection was performed at 360 nm wavelength.

Method development by QbD approach

The goals of HPLC method development have to be clearly defined, as pharmaceutical QbD is a systematic, scientific, risk based, holistic and proactive approach that begins with predefined objectives and emphasizes product and process understanding and control. The ultimate goal of the analytical method is to separate and quantify the main compound.

An experimental design comprised of a standard set of 2 columns, 2 flows, and 7 mobile phase was developed. This led to a total of 28 (2 columns x 2 flows x 7 mobile phases) chromatographic conditions. For each column/ flow/pH/organic modifier combination, a 10 run time was there.

The 28 method conditions were evaluated using the three tiered approach. At the first level, the conditions were evaluated for peaks symmetry, peaks fronting, peaks tailing, and pressure in the system and retention times. This resulted in 10 chromatographic conditions. At the second level, these 10 conditions were further evaluated by using more stringent criteria, biggest peak area and peak height (that will influence the sensibility of the method). As the final method is selected against method attributes,

Parameters	Description of parameters
Columns	Eclipse XDB C18 (150 mm x 4.6 mm, 5 μm) Zorbax SB C18 (150 mm x 3 mm, 3.5 μm)
Mobile phases (sodium acetate buffer, pH adjusted with phosphoric acid)	Water / acetonitrile / methanol (40 / 60 / 0) Water / acetonitrile / methanol (40 / 0 / 60) Water / acetonitrile / methanol (40 / 50 / 10) Water / acetonitrile / methanol (40 / 40 / 20) Water / acetonitrile / methanol (40 / 30 / 30) Buffer pH = 3 / acetonitrile / methanol (40 / 30 / 30) Buffer pH = 5 / acetonitrile / methanol (40 / 30 / 30)
Flows (mL/min)	0.5 0.7

Table 1
EXAMINATION OF THREE PARAMETERS OF HPL

it is highly likely that the selected method is reliable and will remain operational over the lifetime of product.

The optimized HPLC method was validated in accordance with the ICH guidelines (ICH guideline Q2 (R1), for linearity, limits of detection and quantification, system precision, intra- day (method) precision, inter-day (intermediate) precision and accuracy [40-46].

Results and discussions

Method development for column selection

Observation and remarks are shown in table 2.

Taking into account the obtained results, in the subsequent determinations the Eclipse XDB C18 (150 mm x 4.6 mm, 5 μm) column was used.

Method development for flow selection

Observation and remarks are shown in table 3.

Taking into account the obtained results, in the subsequent determinations a flow rate of 0.7 mL/min was used.

Method development for Eclipse XDB column using different mobile phase compositions

If the water content is kept constant at 40% and the acetonitrile / methanol ratio is changed from 60 / 0 to 0 / 60 in steps of 10%, the following remark can be made: (1) irrespective of the ratio of acetonitrile / methanol, the position of the maximum absorption around 360 nm is constant; (2) as the content in methanol increases, the position of the maximum absorption gradually moves to higher wavelengths, the absorbance and the peak height decrease; (3) the retention times increase; (4) peaks area increase; (5) peak height decrease.

If, instead of water, in the mobile phase composition was used a sodium acetate buffer, the retention times decrease (which means a shorter analysis time), peak area is relatively constant but the peak height increase and peak width decrease. Smaller differences was observed between sodium acetate buffer with *p*H = 5 and *p*H = 3.

Taking into account the obtained results, in the subsequent determinations the composition of the mobile phase was chose to be buffer (*p*H = 5)/acetonitrile/ methanol (40/30/30).

Final conditions

The final conditions for separation and quantification of piroxicam from serum are: 20 mL from methanolic supernatant obtained after protein precipitation is injected onto a Eclipse XDB C18 (150 mm x 4.6 mm, 5 μm) column, using a mobile phase consisting from a mixture of sodium acetate buffer (*p*H = 5)/acetonitrile/methanol (40/30/30) at a flow rate of 0.7 mL/min and detection at 360 nm. The retention time for piroxicam is about 2.4 min. Figure 2 shows two chromatograms (for a standard and a sample solution containing piroxicam).

Method validation

Linearity

For the response linearity study three sets of working solutions in a concentration range of 0.5 - 10 μg/mL were prepared. Each solution was analyzed under the optimized conditions; from the obtained chromatograms the peak area for piroxicam was measured. The mean peak area

Table 2
OBSERVATION AND REMARKS OF METHOD DEVELOPMENT FOR COLUMN SELECTION

Column	Observation	Remarks
Eclipse XDB C18	low pressure and retention times	satisfactory
Zorbax SB C18	high pressure and retention times	not satisfactory

Table 3
OBSERVATION AND REMARKS OF METHOD DEVELOPMENT FOR FLOW SELECTION

Flow (mL/min)	Observation	Remarks
0.5	greater retention times and peak width, lower symmetry and peak height	not satisfactory
0.7	smaller retention times and peak width, better symmetry and higher peak height	satisfactory

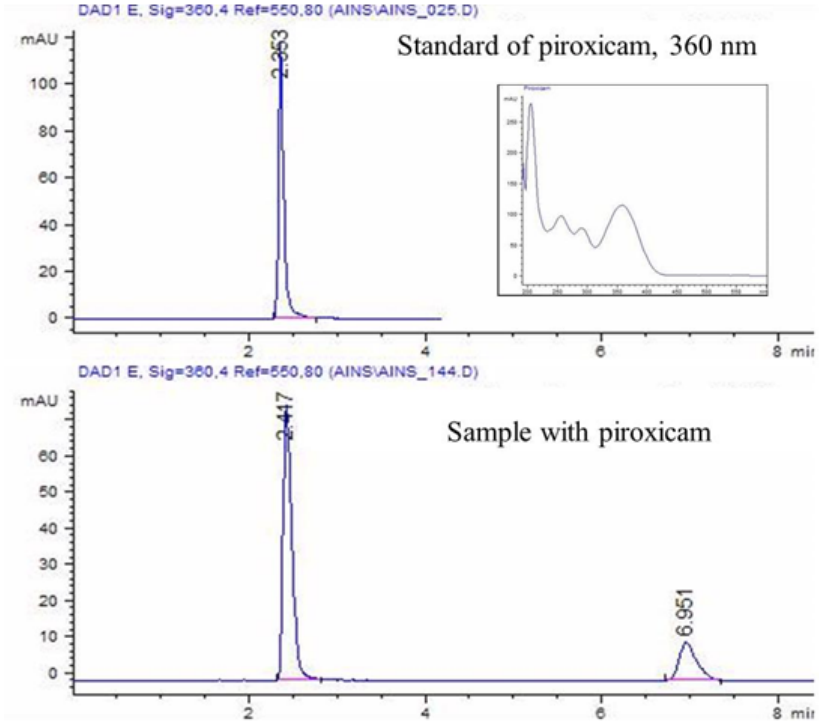

Fig. 2. Chromatograms of standard and sample solutions containing piroxicam

for each concentration was graphically represented (fig. 3) and the equation of the straight line and the regression coefficient were calculated by least squares regression method. In the concentration range 0.5 to 10 μg/mL the regression equation was *Peak area = 22.316 x Concentration – 0.8752* (r^2 = 0.9994).

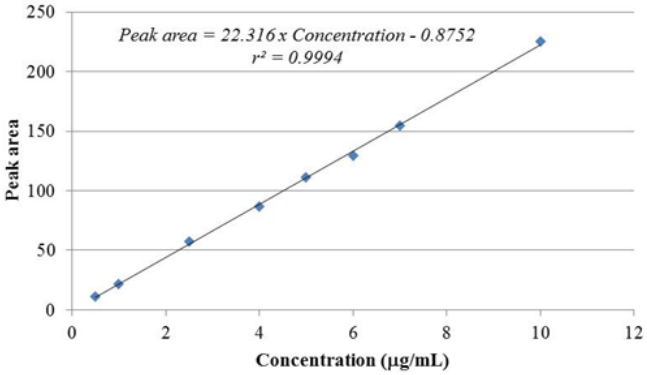

Fig. 3. Response linearity in the range 0.5 - 10 μg/mL

Table 4
SYSTEM PRECISION FOR THE DETERMINATION OF PIROXICAM BY HPLC

No.	Peak area	No.	Peak area
1	112.77	6	108.09
2	108.87	7	110.52
3	110.89	8	108.15
4	112.25	9	109.86
5	112.06	10	108.94
Statistics	Mean = 110.24; SD = 1.7306; RSD = 1.5699%.		

Limit of detection and the limit of quantification

Limit of detection (LOD) and limit of quantification (LOQ) were calculated using standard deviation and regression slope (LOD = 3.3 x SD/slope = 3.3 x 2,4472/ 22.316 = 0.36 μg/mL; LOQ = 10 x SD/slope = 10 x 2,4472/ 22.316 = 1.10 μg/mL).

Precision

To estimate precision, the system precision, method precision and intermediate precision were determined. (1) *Injection repeatability (system precision)* was determined for a number of 10 successive measurements of the same sample (5 μg/mL), RSD % being 1.5699 % (maximum limit imposed is 2%) - table 4. (2) *Method precision* was determined by using three independent solutions in the three different concentration levels (4, 5 and 6 μg/mL) for which the RSD % was 3.4964% (maximum limit imposed is 5%) – table 5. (3) *Intermediate precision* was determined using three independent solutions at three different concentration levels (4, 5 and 6μg/mL) for which the RSD % was 3.9917% (maximum limit imposed is 5%) (table 6).

Accuracy

Accuracy reflects the extent to which a measurement is close to the true value. The accuracy of the method for determination of piroxicam was assessed by the addition method. Thus, recovery for three samples at three different concentration levels in the range of 80-120% (4, 5 and 6 μg/mL) were calculated, obtaining a mean recovery of 98.77 % in the range 95.99 - 103.66 %. Since the recovery method has a value within the specified range (± 5%) it follows that the method is accurate (table 7).

No.	Theoretical concentration (μg/mL)	Peak area	Calculated concentration (μg/mL)	%
1	4	85.72	3.88	97.01
2		88.40	4.00	100.01
3		88.01	3.98	99.58
4	5	115.84	5.23	104.60
5		117.73	5.31	106.30
6		111.60	5.04	100.80
7	6	126.65	5.71	95.24
8		132.69	5.99	99.75
9		130.07	5.87	97.80
Statistics			Mean	100.12 %
			SD	3.5006
			RSD %	3.4964 %

Table 5
METHOD PRECISION FOR THE DETERMINATION OF PIROXICAM BY HPLC

No.	Theoretical concentration (μg/mL)	Peak area	Calculated concentration (μg/mL)	%
1	4	85.66	3.88	96.94
2		83.26	3.77	94.25
3		87.27	3.95	98.75
4	5	119.38	5.39	107.77
5		113.31	5.12	102.33
6		107.77	4.87	97.37
7	6	128.85	5.81	96.89
8		129.45	5.84	97.33
9		132.25	5.97	99.42
Statistics			**Mean**	**99.01 %**
			SD	**3.9520**
			RSD %	**3.9917 %**

Table 6
INTERMEDIATE PRECISION FOR THE DETERMINATION OF PIROXICAM BY HPLC

No.	Theoretical concentration (µg/mL)	Peak area	Calculated concentration (µg/mL)	%
1	4	87.84	3.98	99.39
2		88.05	3.98	99.61
3		85.11	3.85	96.32
4	5	114.79	5.18	103.66
5		111.07	5.02	100.33
6		109.85	4.96	99.23
7	6	130.24	5.88	97.92
8		127.65	5.76	95.99
9		128.25	5.79	96.44
Statistics			**Mean recovery**	***98.77 %***
			Minimum	***95.99 %***
			Maximum	***103.66 %***

Table 7
ACURACY FOR THE DETERMINATION OF PIROXICAM BY HPLC

Conclusions

The developed and validated HPLC method is simple, linear, precise, and accurate, and was successfully applied to determine piroxicam in serum, being sufficiently sensitive. The proposed method can be used in routine analysis of serum samples containing piroxicam.

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