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Statistical experiment design optimization and analytical validation based on total error measurement of a rapid RP-HPLC method for the determination of paracetamol and caffeine in pharmaceutical formulations

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ABSTRACT

This paper describes the optimization and validation of an analytical method for the determination of paracetamol and caffeine in tablets by HPLC using Box Behnken design.

This multivariate approach allows a considerable improvement in chromatographic performance using fewer experiments, without additional cost for columns or other equipment. By applying the quadratic regression analysis, the equations describing the behaviors of the response as simultaneous functions of the selected independent variables were developed. Accordingly, the optimal conditions were determined.

A novel validation strategy based on the accuracy profiles was used to select the most appropriate regression model, to assess the method accuracy within well defined acceptance limits and to determine the limits of quantitation as well as the concentration range.

The statistical methodology allowing to correctly concluding about the validity of a procedure is proposed in this article. Indeed all the steps to obtain the decision tool namely the accuracy profile are described and illustrated. This tool, based on the concept of total error (bias + standard deviation) build with a β-expectation tolerance interval, allows to easily taking the right decision and simultaneously minimizing the risk of the future use of this analytical procedure. Finally, uncertainty derived from β-expectation tolerance interval, which is equal to the uncertainty of measurements as well as the expanded uncertainty using a coverage factor k= 2 was estimated.

KEYWORDS

Paracetamol;
Caffeine;
Validation;
Accuracy profile;
Total error;
Uncertainty;
Pharmaceutical formulations,
RP-HPLC.

INTRODUCTION

Paracetamol (acetaminophen, N-acetyl-p-aminophenol, 4-acetamidophenol) is one of the popular non-steroidal anti-inflammatory drugs widely used for management of pain and fever in a variety of patients including children, pregnant women, the elderly and those osteoarthritis, simple headaches and

non-inflammatory musculoskeletal conditions.

Paracetamol is used as analgesic and antipyretic agents. Its action is similar to aspirin, and is a suitable alternative for patients who are sensitive to aspirin.

Caffeine (3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione) is an alkaloid N-methyl derivative of xanthine widely distributed in natural products, commonly used in beverages.

It has many physiological effects, such as gastric acid secretion, diuresis, and stimulation of the central nervous system[1]. Caffeine is used therapeutically in combination with ergotamine in the treatment of migraine or in combination with no steroidal anti-inflammatory drugs in analgesic formulations.

Paracetamol and caffeine appear to be associated in many commercial formulations because caffeine increases the analgesic character of paracetamol[1].

Numerous methods have been reported for the determination of paracetamol and caffeine including spectrophotometric[2-4], chromatographic[5-14], infra red[15,16], flow-injection[17], electrochemical[18-23], spectrofluorimetric[24-27], and chemiluminescent[28-29].

The majorities of methods used in routine control laboratory have poor selectivity and do not take into account also the impurity k (4-aminophenol) of paracetamol. Indeed, the peak of 4-aminophenol may interfere with that of paracetamol or that of caffeine so these methods have poor separation on the one hand between the peak of paracetamol and the peak of caffeine and other hand between the peak of paracetamol and the peak of 4-aminophenol which generates a bad determination of two drugs in pharmaceutical forms. Hence the need to optimize the operating conditions chromatographic to have a good selectivity.

Developing and optimizing an isocratic HPLC method is a complex procedure that requires simultaneous determination of several factors (e.g. type and composition of the organic phase, column temperature, flow rate, pH, type of the stationary phase, etc.).

The principles behind these techniques (known Response surface methodology), encompasses the use of experimental design, generation of mathematical equations and graphic outcomes.

Response surface methodology (RSM) is a collection of statistical and mathematical techniques used for the improvement and optimization of complex processes[30,31]. It is a commonly used method to find the optimal chromatography conditions for the separation of the drug compounds. The model equation easily clarifies the effects for binary combination of the independent parameters. In addition, the empirical model that related the response to the independent variable is used to obtain information about the process. With respect to these, one can say that RSM is a useful tool for the optimization. Finally, RSM also represents a more economical approach as the number of experiments can be significantly reduced.

The aim of the present paper was to develop and optimize a simple and rapid high-performance liquid chromatography method for the simultaneous determination of paracetamol and caffeine, using experimental design. The significance of the studied factors was evaluated with the optimum chromatographic conditions were estimated by a Box Behnken design (BBD) using both a graphical (response surface and overlay contour plots) and a mathematical (Derringer's desirability function) global optimization approach. Finally, the proposed method has been fully validated according to the new strategy proposed by the Commission of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) for the validation of quantitative analytical procedure[32-36]. All the statistical calculations necessary to implement the concepts presented in the SFSTP guide are developed in this paper (see appendix[37]) for the quantitative determination of paracetamol and caffeine in pharmaceutical preparations.

EXPERIMENTAL

Chemicals and solvents

All chemicals and solvents used were of analytical or HPLC grade. Paracetamol and caffeine acid were supplied by the European Pharmacopoeia (Strasbourg, France). Potassium dihydrogen phosphate, sodium octanesulfonate, 4-aminophenol were purchased from Acros Organics (Geel, Belgium). Phosphoricacid (85%) was obtained from Merck (Darmstadt, Germany). Deionized water was generated from Milli-Q water purifying system (Millipore, Watford, UK). Phosphate buffer solution was prepared by dissolving 6.8 g of

potassium dihydrogen phosphateand 1,17g of sodium octansulfonate in 1.l of deionized water. The pH was adjusted to 4.5 with phosphoric acid.

Apparatus

The method development was performed with a *PERKIN ELMER* LC system with a model series 200 pump, a model series 200 injector and a model series 200 DAD detector. The system was controlled and data analyses were performed with the TotalChrom software version 5.2

Chromatographic conditions

The chromatographic analysis was performed on a Lichrospher 100 RP-18 column (250mmx4mm i.d, 5µm)and kept at 25 æ%C. The mobile phase was prepared by mixing acetonitrile and phosphate buffer solution (pH 4.5) in a ratio 15:85 (v/v) and was degassed before use. The HPLC system was operated isocratically at a flow rate of 1 ml/min and the injection volume was 20µl. UV detection was performed at 270 nm and peaks were identified with retention times and UV spectra.

Standard solutions

(a) Solutions used for method development

Paracetamol (500 mg) and caffeine (50 mg) were accurately weighed in a 50ml volumetric flask and dissolved in the mobile phase and filled up to volume with same solvent. This stock solution was degassed in an ultrasonic bath for at least 10min. after that, subsequent dilutions were performed in order to obtain several solutions at the concentration levels as mentioned in TABLE 1.

(b) Solutions used for method validation and routine

Independent stock solutions of paracetamol and caffeine were prepared in the same way as mentioned in point Section 2.5.1. Subsequent dilutions were carried out in order to obtain the calibration and validation standards. Six concentrations (m=6) of paracetamol and of caffeine were used. Each concentration was analyzed 3 times (n=3) for 3 days (k=3). The experimental design is illustrated in TABLE 1.

Computations

Work on experimental design, data analysis, response surfaces, contour diagrams and the regression models were carried out using the software Minitab®15. The accuracy profiles were developed using the Microsoft office Excel version2010.

RESULTS AND DISCUSSION

Method development

(a) Box-Behnken experimental design

In order to study the simultaneous variation of the factors on the analytical responses, a multivariate approach usinga Box–Behnken statistical experimental designwas applied. This design was constructed based on a 3^3 factorial design, three replications of the central run, leading to 15 sets of experiments, allowing each experimental response to be optimized.

Before starting an optimization procedure, it is important to identify the crucial factors affecting the quality of the derived outcomes. The three factors evaluated in this design and their levels were reported in TABLE 1. All other factors such as the temperature of column thermostatisation, the stationary phase, the injection volume and the wavelength of detection were maintained constant.

A three-factor, three-level Box–Behnken design was applied for the optimization procedure and the analytical responses used in this experimental design were resolution (R_{S1}) between PAC and AMP peaks (R_{S1}>2), resolution (R_{S2}) between AMP and CAF peaks (R_{S2}>2) and retention time (R_T) of PAC (R_T>4min).

TABLE 1 : The levels of the variables chosen for the trials

Independent factors	Unit	Symbol	Levels		
			Low	Middle	High
Flow rate	Ml min^{-1}	X1	0,8	1,4	2
Proportion of CAN	%	X2	10	15	20
Ph	-	X3	3	5	7

(b) Statistical analysis

Regression analysis was performed for the experiment data and was fitted into the empirical second order polynomial model, as shown in the following equation:

$$Y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{12}x_{12} + a_{13}x_{13} + a_{23}x_{23} + a_{11}x^2_1 + a_{22}x^2_2 + a_{33}x^2_3 + \varepsilon$$

Where Y is the response calculated by the model; X_1,

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X_2 and X_3 are coded variables, corresponding to flow rate, pro.ACN and pH, respectively. a_1, a_2 and a_3 are the linear; a_{11}, a_{22} and a_{33} are the quadratic and a_{12}, a_{13} and a_{23} are the cross-product effects of the X_1, X_2 and X_3 factors on the response.

The quality of the fitted model was expressed by the coefficient of determination R^2, and its statistical significance was checked by an F-test (analysis of variance) at the 5% significance level.

The statistical significance of the regression coefficients was determined by using the t-test (only significant coefficients with *p*-value < 0.05 are included).

The analysis of variance for the experimental results of the Box–Behnken design is also shown in TABLE 2. The coefficient of determination (R^2) of the model was about 0.98 for the three responses, indicating that the model adequately represented the real relationship between the parameters chosen. Furthermore, results of the error analysis indicated that the lack of fit was insignificant (p-value> 0.05).

TABLE 2 : ANOVA of response surface quadratic model

Source	Resolution-1			Resolution-2			Time retention		
	MS	F	p-value	MS	F	p-value	MS	F	p-value
Regression	1.913	25.00	0.001	1.473	28.60	0.001	1.562	40.57	0.000
Residual Error	0.076			0.052			0.038		
Lack-of-Fit	0.121	12.08	0.077	0.079	7.92	0.114	0.057	5.75	0.152
Pure Error	0.010			0.010			0.010		
R^2	0,978			0,981			0,987		

The regression coefficients of the intercept, linear, quadratic, and interaction terms of the model were calculated using the least square technique and are presented in TABLE 3. It was evident that all the linear parameters and quadratic parameters were found to be significant ($p < 0.05$), whereas all the interaction parameters were insignificant ($p > 0.05$).

$$\mathbf{Y_1 = 3.90 + 0.61\ X_1 + 0.83\ X_2 + 0.86\ X_3 - 0.65\ X^2_1 - 0.43\ X^2_2 - 0.50\ X^2_3} \quad \mathbf{(1)}$$

$$\mathbf{Y_2 = 4{,}9 + 0{,}46\ X_1 + 0.73\ X_2 + 0.79\ X_3 - 0.49\ X^2_1 - 0{,}46\ X^2_2 - 0{,}49\ X^2_3} \quad \mathbf{(2)}$$

$$\mathbf{Y_3 = 5.5 + 0.41\ X_1 + 0.51\ X_2 + 1.03\ X_3 - 0.58\ X^2_1 - 0.28\ X^2_2 - 0.45\ X^2_3} \quad \mathbf{(3)}$$

TABLE 3 : Regression coefficients and their significance in the quadratic model of resolution and retention time

Terms	Resolution-1			Resolution-2			Time retention		
	Estimate	t-test	p-value	Estimate	t-test	p-value	Estimate	t-test	p-value
Constant	3.90	24.42	0.000	4.90	37.39	0.000	5.500	48.55	0.000
Flow rate	0.613	6.26	0.002	0.463	5.76	0.002	0.413	5.95	0.002
Pro. CAN	0.825	8.44	0.000	0.725	9.04	0.000	0.513	7.39	0.001
Ph	0.863	8.82	0.000	0.787	9.82	0.000	1.025	14.78	0.000
Flow rate*Flow rate	-0.650	-4.52	0.006	-0.487	-4.13	0.009	-0.575	-5.63	0.002
Pro. ACN*Pro. ACN	-0.425	-2.95	0.032	-0.463	-3.92	0.011	-0.275	-2.69	0.043
pH*pH	-0.500	-3.47	0.018	-0.487	-4.13	0.009	-0.450	-4.41	0.007
Flow rate*Pro. ACN	0.025	0.18	0.864	0.200	1.76	0.138	-0.000	-0.00	1.000
Flow rate*pH	0.100	0.72	0.502	-0.025	-0.22	0.834	0.025	0.26	0.809
Pro. ACN*pH	-0.075	-0.54	0.611	0.050	0.44	0.678	-0.225	-2.29	0.070

(c) Response surface optimization

The optimum processing conditions were obtained by using graphical and numerical analysis based on the criterion of desirability function and the response surface. Derringer's desirability function (D) can take values from 0 to 1. A value close to unity indicates that the combination of the different criteria is matched in a global optimum. The desirability was close to 0.88 when the mobile phase flow was 0.97 mL min^{-1}, the proportion of acetonitrile was 17.4%, and the pH was 4.1. To facilitate interpretation of the results, we decided to fix the flow rate at 1 mL min^{-1}.

Figure 1a and 1b shows the effect of pH and proportion of ACN on resolution-1 and resolution-2 at a constant flow rate of 1 mL min^{-1}. Indeed, the resolution-1 and resolution-2increased when pH and % acetonitrile concentration increased, especially from pH 4.0 and proportion of ACN 14.5%. Figure 1c shows the effect of pH and proportion of ACN on retention time of PAC at fixed flow rate, especially from pH 4.0 and proportion of ACN 14.0%.

The contour plot of the responses in Figure 2 shows the zone of optimization (colorless zone: R_{S1}, R_{S2} > 2 and R_T > 4) and describes pH and proportion of ACN to be in the ranges 3.5– 5.0 and 13.0–16.5, respectively.

Finally, taking into account the factor values obtained from the desirability function and the surfaces of the responses. The optimal operating conditions of the chromatographic method for simultaneous determination of PAC and CAF are 1mL min^{-1}, 4.5 and 15% of the mobile phase flow rate, pH and amount of acetonitrile, respectively.

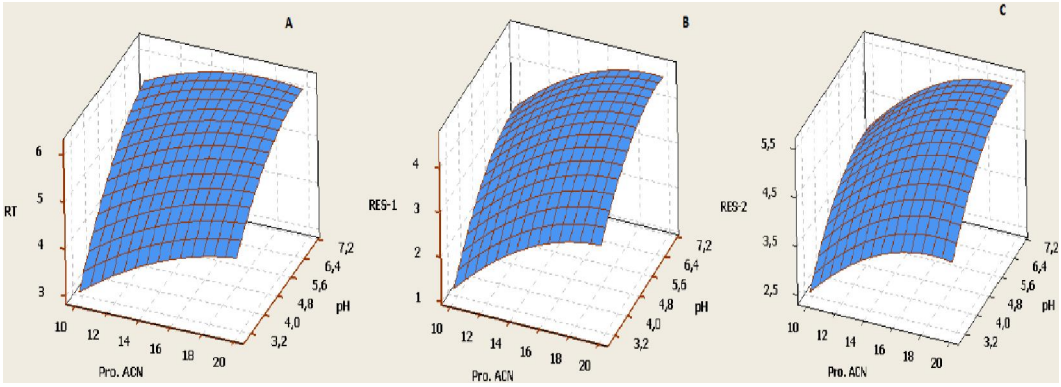

Figure 1 : Response-surface plots representing the effect of mobile phase pH and proportion of acetonitrile on the responses: (a) retention time of paracetamol, (b) resolution-1 and (b) resolution -2. Mobile phase flow rate was constant at 1 mL min-1

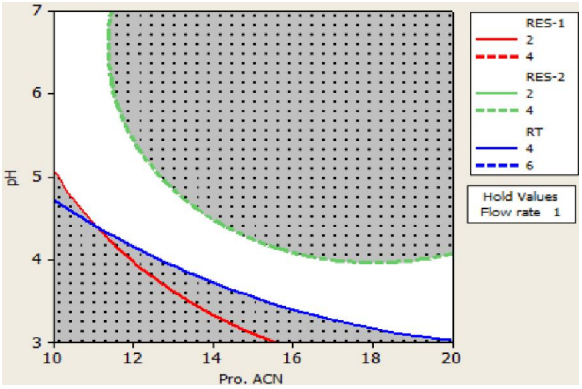

Figure 2 : The contour plot of the resolution and the retention time of paracetamol for proportion of acetonitrile and mobile phase pH. Mobile phase flow rate was kept constant at 1 mL min-1

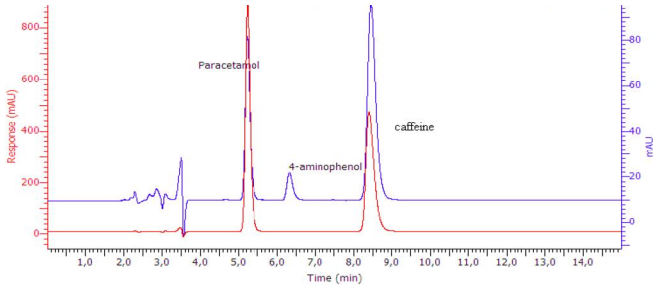

Figure 3 : chromatogram of the mixture of paracetamol, 4-aminophenol and caffeine

The chromatogram obtained by use of these conditions is shown in Figure 3. It is apparent response surface predictions were in good agreement with the experimental results. Therefore, Box– Behnken statistical design was reliable and effective in determining the optimum conditions.

Validation

(a) Selectivity

The method selectivity was checked by comparison of typical chromatograms obtained by injecting a blank-mobile phase and solution of the placebo and solution of paracetamol, caffeine and 4-aminophenol. No interfering endogenous peak could be detected as shown in the chromatogram of blank-mobile phase and of the placebo (figure 4 & figure 5). In addition, as can be seen in figure 3 the peak corresponding to paracetamol can be easily integrated in presence of the peak corresponding to 4-aminophenol. The retentions times were 5.25, 6.3 and 8.5 of paracetamol, 4-aminophenol and caffeine respectively.

Figure 4 : Superposition the chromatogram of the solution of paracetamol and caffeine with chromato-

gram of mobile phase

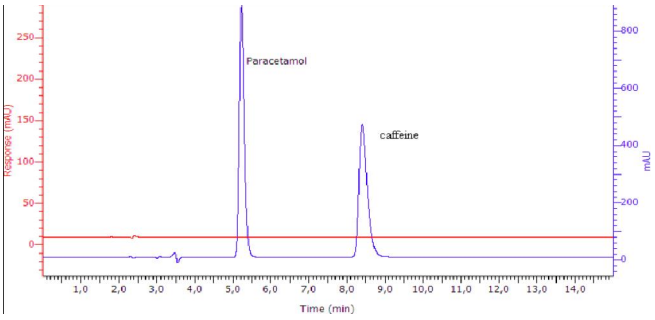

Figure 4 : Superposition the chromatogram of the solution of paracetamol and caffeine with chromatogram of mobile phase

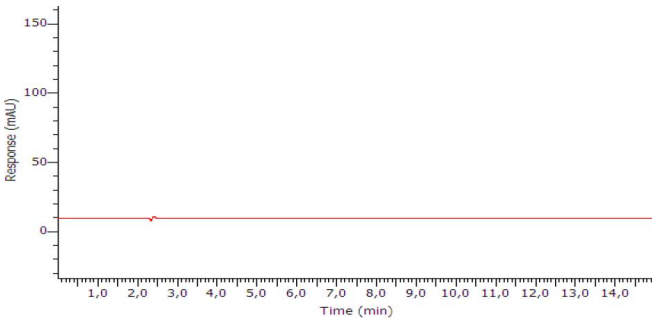

Figure 5 : chromatogram of the placebo

<Figure 5>

(b) Response function

The response function of an analytical procedure is, within the range selected, the existing relationship between the response (signal) and the concentration (quantity) of the analyte in the sample. In the present study, eight different response functions were tested (for paracetamol and caffeine) and accuracy profiles build for each of them. Five of the tested models appear to answer the objective of determination of paracetamol: the quadratic regression model, the linear regression after square root transformation, the linear regression model, the weighted $1/X$ linear model and the weighted $1/X^2$ linear regression model. For the quantification of caffeine, five calibrations models can be used to describe adequately the relationship between concentration and analytical response: the linear regression model, the linear regression after square root transformation, the weighted $1/X$ linear model, the weighted $1/X^2$ linear model and the linear regression after logarithm transformation. However, their application in routine can be quite long and not easy. Therefore, we selected the simple linear model which firstly responds to the objectives of our method and also is convenient for routine analysis.

Concentrations recovered from the validation standards are calculated from the simple linear model, which yielded for each level of concentration mean relative bias, the tolerance upper and lower the expected values at the β and this, in considering the standard deviation of intermediate precision. Then, the accuracy profile of paracetamol and caffeine are constructed from these data. See Figure6 & 7.

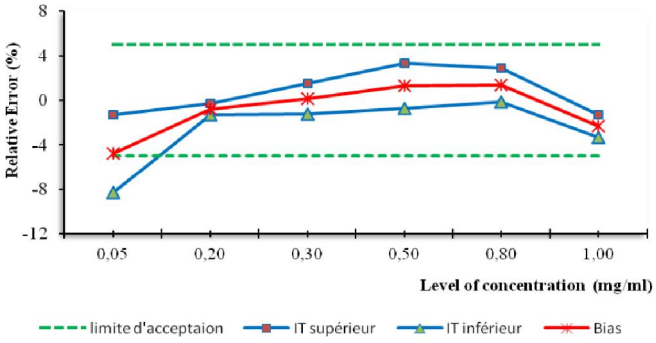

Figure 6 : Accuracy profiles of paracetamol using linear regression model

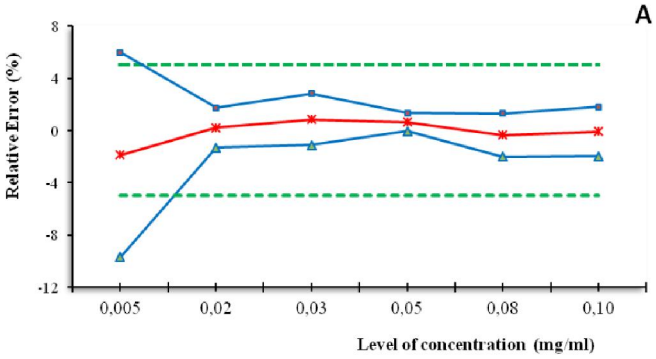

Figure 7 : Accuracy profiles of caffeine using linear regression model

(c) Precision

Precision is the closeness of agreement among measurements from multiple sampling of a homogeneous sample under the recommended conditions. It gives some information on random errors and it can be evaluated at two levels: repeatability and intermediate precision. Results are presented in TABLES 2 & 3

The R.S.D. values mentioned in TABLES 2 & 3 were relatively low; the relative standard deviation values for repeatability and intermediate precision were between (0.87, 0.89% for paracetamol) and (0.68, 2.95% for caffeine), illustrating the good precision of the proposed method.

(d) Trueness

Trueness refers to the closeness of agreement be-

tween a conventionally accepted value and a mean experimental one. It gives information on systematic error.

TABLES 4 & 5 report trueness expressed as relative bias and recovery for the different level of validation standards. Trueness was acceptable for the two analyzed actives substances, since the bias did not exceed the value of ± 5%, irrespective of the concentration level, except at the lowest concentration level of paracetamol.

TABLE 4 : Validation results for paracetamol using the linear regression model

Validation criterion for paracetamol				
		Day 1	Day 2	Day 3
Response function (p = 3; n = 3; m = 6)	Slope	15250275	15333603	15282501
	Intercept	227713	225633	215965
	r^2	0,9977	0,9983	0,9975
Trueness (p = 3; n =3)	**Relative bias (%)**		**Recovery (%)**	
0,05 (mg/ml)	-21,11		78,89	
0,20 (mg/ml)	-2,43		97,57	
0,30 (mg/ml)	0,13		100,13	
0,50 (mg/ml)	1,32		101,32	
0,80 (mg/ml)	1,38		101,38	
1,00 (mg/ml)	-2,81		97,19	
Precision	**Repeatability (%)**		**Intermediate precision (%)**	
0,05 (mg/ml)	0,64		0,89	
0,20 (mg/ml)	0,10		0,47	
0,30 (mg/ml)	0,54		0,56	
0,50 (mg/ml)	0,22		0,55	
0,80 (mg/ml)	0,87		0,87	
1,00 (mg/ml)	0,39		0,53	
Accuracy (p = 3; n = 3)	**Relative β-expectation tolerance limit (%)**			
	Lower tolerance		**Upper tolerance**	
0,05 (mg/ml)	-23,7		-18,6	
0,20 (mg/ml)	-4,5		-0,4	
0,30 (mg/ml)	-1,62		1,9	
0,50 (mg/ml)	-0,9		3,6	
0,80 (mg/ml)	-0,7		3,5	
1,00 (mg/ml)	-4,3		-1,3	
Linearity (p = 3; n = 3; m = 5)				
Range (mg/l)	[0,2-1]			
Slope	0,9813			
Intercept	0,0075			
r^2	0,998			

p: number of series of analysis; n: number of replicates per series; m: numbers of concentration levels.

TABLE 5 : Validation results for caffeine using the linear regression model

Validation criterion for caffeine				
		Day 1	Day 2	Day 3
Response function (p = 3; n = 3; m = 6)	Slope	56701470	56521755	56525337
	Intercept	-9949	3467	2194
	r^2	0,9999	0,9999	0,9999
Trueness (p = 3; n = 3)	**Relative bias (%)**		**Recovery (%)**	
0,005 (mg/ml)	3,75		103,75	
0,020 (mg/ml)	0,21		100,21	
0,030 (mg/ml)	0,86		100,86	
0,050 (mg/ml)	0,64		100,64	
0,080 (mg/ml)	-0,36		99,64	
0,1 (mg/ml)	-0,09		99,91	
Precision	**Repeatability (%)**		**Intermediate precision (%)**	
0,005 (mg/ml)	0,64		2,95	
0,020 (mg/ml)	0,30		0,50	
0,030 (mg/ml)	0,63		0,76	
0,050 (mg/ml)	0,23		0,27	
0,080 (mg/ml)	0,68		0,68	
0,1 (mg/ml))	0,08		0,44	
Accuracy (p = 3; n = 3)	**Relative β-expectation tolerance limit (%)**			
	Lower tolerance		**Upper tolerance**	
0,005 (mg/ml)	-9,2		16,7	
0,020 (mg/ml)	-1,3		1,7	
0,030 (mg/ml)	-1,11		2,8	
0,050 (mg/ml)	-0,1		1,4	
0,080 (mg/ml)	-2,0		1,3	
0,1 (mg/ml)	-2,0		1,9	
Linearity (p = 3; n = 3; m = 5)				
Range (mg/l)	[0,02-0,1]			
Slope	0,9954			
Intercept	0,0003			
r^2	0,9999			

p: number of series of analysis; n: number of replicates per series; m: numbers of concentration levels.

(e) Accuracy

Accuracy refers to closeness of agreement between the test result and the accepted reference value, namely the conventionally true value. The accuracy takes into account the total error, i.e. the sum of systematic and random errors, related to the test result. As shown in TABLES 4 & 5, the upper and the lower β-expectation tolerance limits of the mean bias (%) did not ex-

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ceed the acceptance limits settled at 5% for each concentration level (except at the lowest concentration level of the two actives substances). Consequently, the method can be considered as accurate over the concentration range investigated.

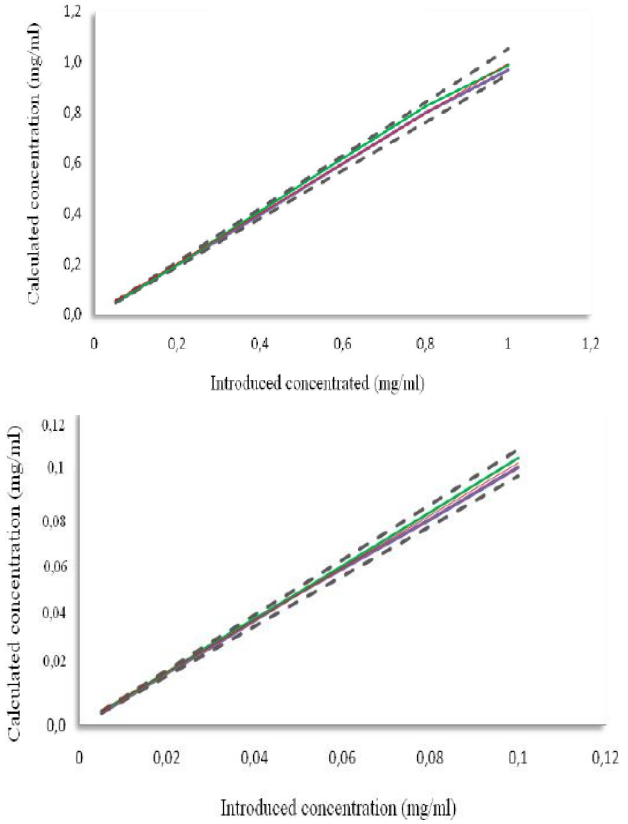

Figure 8 : linearity profiles for paracetamol and caffeine. The red line is the identity line (Y=X), the green and blue lines are the upper and lower β-expectation tolerance limits and the dashed lines are the upper and lower acceptance limits

(f) Linearity

The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentration (quantities) of analyte in the sample. In order to demonstrate method linearity, a regression line was fitted on the calculated concentrations of the validation standards as a function of the introduced concentrations by applying a linear regression model. The equations obtained for paracetamol and caffeine with their coefficient of determination are presented in TABLES 4 & 5.

The linearity of the method was demonstrated using the â-expectation tolerance interval approach. Indeed, as illustrated in figure 8, the upper and lower β-expectation tolerance limits were included inside the absolute acceptance limits irrespective to the concentration levels for the two analytes studied.

(g) Uncertainty of measurement

The uncertainty characterizes the dispersion of the values that could reasonably be attributed to the measurand, i.e. the concentration of paracetamol and caffeine in our study. Several uncertainty results were generated and are presented in TABLE 6. The expanded uncertainty was computed using a coverage factor of k = 2, representing an interval around the results where the unknown "true value" can be observed with a confidence level of 95%. As shown in TABLE 6, the relative expanded uncertainty of paracetamol and caffeine irrespective of the concentration levels did not exceed 5%, except at the lowest concentration level of caffeine.

TABLE 6 : Estimates of the measurement uncertainties related to paracetamol and caffeine, at each concentration level investigated in validation using the selected regression models

Analyte	Concentration (mg/ml)	Uncertainty of the bias (mg/ml)	Uncertainty (mg/ml)	Expanded Uncertainty (mg/ml)	Relative expanded uncertainty (%)
Paracetamol	0,05	2,09E-04	4,93E-04	9,87E-04	1,97
	0,20	5,38E-04	1,09E-03	2,18E-03	1,09
	0,30	5,97E-04	1,77E-03	3,55E-03	1,18
	0,50	1,50E-03	3,13E-03	6,26E-03	1,25
	0,80	2,32E-03	7,34E-03	1,47E-02	1,83
	1,00	2,43E-03	5,80E-03	1,16E-02	1,16
Caffeine	0,005	8,39E-05	1,70E-04	3,40E-04	6,79
	0,02	4,98E-05	1,11E-04	2,22E-04	1,11
	0,03	9,79E-05	2,49E-04	4,97E-04	1,66
	0,05	5,70E-05	1,49E-04	2,97E-04	0,59
	0,08	1,83E-04	5,75E-04	1,15E-03	1,44
	0,1	2,53E-04	5,09E-04	1,02E-03	1,02

CONCLUSION

In this article, a novel validation strategy based on the accuracy profiles was successfully applied to demonstrate the capacity of the HPLC method for simultaneous determination of paracetamol and caffeine in pharmaceutical formulations. An original validation approach using accuracy profiles based on β-expectation tolerance intervals for the total measurement error permit to indicate the capability of the method. The concept of accuracy profile was also used to select the most appropriate regression model for calibration, to determine the range of which the method can be considered as valid.

Furthermore, the measurements uncertainties were estimated without any additional experiments thanks to the validation methodology, allowing correct interpretation and comparison of the results in a cost effective procedure.

Appendix

A.1 Building accuracy profile

The total error of analytical measurement is the simultaneous combination of systematic and random error. Systematic error is measured by a bias $\hat{\mu}_j$ and random error by a variance $\hat{\sigma}^2$

One way to estimate this total error is to compute the ²-expectation tolerance interval introduced by Mee[38], and to compare it to acceptance limits ». The equation of the ²-expectation tolerance interval is:

$$\left[\hat{\mu}_j - Q_t\left[\nu, \frac{1+\beta}{2}\right]\sqrt{1+\frac{1}{pnB_j^2}}\,\hat{\sigma}_{IP,j};\ \hat{\mu}_j + Q_t\left[\nu, \frac{1+\beta}{2}\right]\sqrt{1+\frac{1}{pnB_j^2}}\,\hat{\sigma}_{IP,j}\right]$$

Where:

- $\hat{\mu}_j$ is the estimate of the mean results of the jth concentration level
- $Q_t\left[\nu; \frac{1+\beta}{2}\right]$ is the ² quantile of the student t distribution with ½ degrees of freedom
- p is the number of series.
- n is the number of independent replicates per series
- $\hat{\sigma}^2_{IP} = \hat{\sigma}^2_{w,j} + \hat{\sigma}^2_{B,j}$ is the estimate of the intermediate precision variance at the jth concentration level, which is the sum of the within series variance $\hat{\sigma}^2_{w,j}$ and the between series variance $\hat{\sigma}^2_{B,j}$
- $R_j = \frac{\hat{\sigma}^2_{B,j}}{\hat{\sigma}^2_{W,j}}$
- $B_j = \sqrt{\frac{R_j+1}{nR_j+1}}$
- $\nu = \frac{(R+1)^2}{\frac{\left(R+\frac{1}{n}\right)^2}{p-1} + \frac{1-\frac{1}{n}}{pn}}$

A.2 Measurement uncertainty

The measurement uncertainty $\hat{\sigma}^2_x$ of a result x is estimated by:

$$\hat{\sigma}^2_x = \hat{\sigma}^2_{IP} + \hat{\sigma}^2_{\hat{\mu}}$$

Where:

- $\hat{\sigma}^2_{IP}$ is the estimated intermediate precision standard deviation.
- $\hat{\sigma}_{\hat{\mu}} = \sqrt{\frac{\hat{\sigma}^2_{IP}\left(1-\phi+\left(\phi/n\right)\right)}{p}}$

  Where $\hat{\sigma}_{\hat{\mu}}$ is the uncertainty associated with the estimator of the bias; $\phi = \frac{\hat{\sigma}^2_w}{\hat{\sigma}^2_{IP}}$ with $\hat{\sigma}^2_w$ being an estimate of the repeatability variance.

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