Full Validation of the Method Employed to Analyze Oxycodone and Imatinib with LC-MS Using Monte-Carlo Simulation

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This work aims to confirm the reliability and applicability of the decision profile as a new overall strategy for the analytical validation and estimation of measurement uncertainty by applying it to the analysis of oxycodone and imatinib using LC-MS. The primary goal of this new approach is to unite two essential criteria prescribed by the ISO IEC 17025, namely analytical validation and estimation of measurement uncertainty, which are required to produce quality results [3]. This innovative approach, recently developed in our laboratory, is defined as a simple and efficient graphical decision-making tool based on the calculation of the tolerance interval of the type "ß-? content" [7,8] using Monte-Carlo simulation. This technique uses the notion of uncertainty instead of accuracy to evaluate the performance of analytical methods, without the need for additional experiments.

Keywords: Analytical validation; Decision profile; Measurement uncertainty; Monte-Carlo simulation; Tolerance interval of type "ß-γ content"

Oxycodone or 4,5a-Epoxy-14-hydroxy-3-methoxy-17-methylmorphinan-6-one (Figure 1) is a semi-synthetic opioid analgesic, derived from thebaine. It is a narcotic analgesic that acts in a similar way to morphine and is proposed for treating the intense chronic pain caused by cancer. Furthermore, oxycodone is used when other lower-level analgesics are not effective or when the patient exhibits intolerable side effects to such drugs.

Oxycodone appears on the list of narcotic drugs, indicating that it acts on the nervous system and is addictive. Therefore, the patient must take it with care, while the number of therapeutic units per dose, the number of doses, and the dosage of the prescribed specialty must be indicated in full[1].

Imatinib or 4-(4-methyl piperazine-1-methyl)-N-4-methyl-3-[4-(3- pyridyl) pyrimidine-2-amino]-benzamide (Figure 2) is a small molecule protein kinase inhibitor that significantly restricts the activity of platelet-derived growth factor (PDGF) tyrosine kinase. It is widely used in the treatment of chronic myeloid leukemia (CML) and gastrointestinal stromal tumors[2].

As noted previously, oxycodone and imatinib are two molecules used in the treatment

of cancer, but at certain doses, their side effects can lead to death[1, 2].

Consequently, it is essential that the analytical results are reliable and accurate, especially when the safety of patients is at stake. This means that the analytical results should reflect the content of the sample as accurately as possible.

ISO/IEC 17025 represents an international standard, which indicates the general requirements for the competence of calibration and testing laboratories. Moreover, this standard applies to all laboratories, regardless of the purpose of their activities, or their size.

Chapter 5 of this standard is crucial and covers the technical requirements for producing quality results, namely, analytical validation and estimation of measurement uncertainty[3].

Analytical validation can be considered crucial as many guides have been published that explain how to validate methods (IUPAC Technical Report: Harmonized guidelines for single-laboratory validation of methods of analysis; ICH Recommendations: Validation of Analytical Procedures: text and methodology; NIST Recommendations: NIST Guidelines for Evaluating and Expressing Uncertainty of NIST Measurement Results; The ISO 5725 Series of Standards; ISO 17025, 2005; ISO 15189, 2012; COFRAC Guides). Unfortunately, these guides often contain inconsistencies in terminology and the statistical methods proposed. In practice, the proposed procedures often present problems in the final decision. To address these issues, in 2003 the SFSTP[4] proposed a new strategy for analytical validation called the accuracy profile. This method suggests creating a simple and efficient graphical decision-making tool based on the tolerance interval "ß-expectation" as a statistical methodology[5]. The advantage of this approach is that it resolves conflicts between accuracy and trueness as previous approaches treat accuracy and trueness independently. This has resulted in ambiguous conclusions that can only be reached if one of these two criteria are met. In contrast, the accuracy profile facilitates the simultaneous representation of both criteria in the same graph.

ISO IEC 17025 is also based on measurement uncertainty. This can be defined as a parameter that characterizes the dispersion of values assigned to a measurand[3]. The term uncertainty has only recently become familiar to chemists. However, it is now considered a key issue in laboratories as it requires a high degree of technicality and the mastery of statistical tools.

The methodology described in the Eurachem Guide[6] for estimating measurement uncertainty is based on revealing the sources of error for each method. These should then be organized in the form of a 5M diagram (material, labor, material, medium, and method). The disadvantages of this methodology are its complexity and high cost.

As such, our laboratory has developed a new strategy for estimating measurement uncertainty and validating analytical methods called the decision profile[7, 8]. This aims to combine these two requirements and facilitate the estimation of measurement uncertainty.

This new approach uses a simple and effective graphical decision-making tool based on the Monte Carlo simulation that calculates the tolerance of interval "ß-? content". It uses the notion of uncertainty instead of accuracy to evaluate the performance of analytical methods.

MATERIALS AND METHODS

Analytical Procedure

To demonstrate the wide applicability of the decision profile when evaluating chromatographic procedures, oxycodone and imatinib in human plasma were analyzed using LC-MS. In addition, a supported liquid extraction (SPE) extraction was used to determine levels of oxycodone and a protein precipitation (PP) extraction was used to determine levels of imatinib[9].

Key information demonstrating the analyte assays is given in Table 1.

Calibration and Validation Protocol

To validate this method for determining levels of oxycodone in human plasma, calibration standard solutions were prepared by doping human plasma with four concentration levels. The lowest concentration level acted as the limit of quantification. Each sample was analyzed six times (n=6) on six different days (p=6). Depending on the choices made, the calibration plan was adopted as $4 \times 6 \times 6 = 144$[9]. Adopting the same calibration plan and the same concentration levels, 144 validation standard solutions were prepared by spiking a

stock solution. This solution was prepared by homogenizing human plasma with oxycodone.

To validate this method for determining levels of imatinib in human plasma, calibration standard solutions were doped with human plasma at four concentration levels. The lowest concentration level acted as the limit of quantification. Each sample was analyzed six times (n=6) on five different days (p=5). Depending on the choices made, the calibration plan was adopted as $4 \times 6 \times 5 = 120$ [9]. To prepare the validation standard solutions, the same concentrations and calibration protocol were chosen as for determining levels of oxycodone. Homogenized human plasma was spiked with imatinib and 120 validation standard solutions were prepared.

Decision Profile

The decision profile is a graphical decision tool that aims to assure laboratories that each measurement will be carried out in a routine manner. Once the procedure has been validated, it will produce measurements sufficiently close to the true unknown value of the sample, or at least within an acceptable limit depending on the purpose of the procedure. This new approach is based on the calculation of tolerance interval type "ß-? content"[7, 8]. It uses the Liao-Lin-Iyer method[11, 12] which is based on the Monte Carlo simulation.

Monte Carlo Simulation

The Monte Carlo simulation is a general method used early on in metrology to estimate measurement uncertainty when the GUM approach is not easily applicable. It artificially reconstructs a random phenomenon by simulating a fictitious realization sample from the input variables. As a result, this method can obtain the distribution of values for the characterized measurand using the distributions of the input quantities. This leads to a level of confidence in the result of the measurement uncertainty estimation.

Therefore, the Monte Carlo method provides a numerical approximation of measurand distribution. Using this method, determining a mean and a standard deviation becomes a simple process[13, 14].

Constructing the Decision Profile

To build the decision profile, the following steps were carried out:
• The acceptance limits were chosen.
• The appropriate response functions were chosen.
• Based on the selected calibration model, the inverse concentrations were calculated.
• The tolerance interval of ß-? for each concentration level was calculated.
• The measurement uncertainty for each level was determined.
• The decision profile was constructed and the 2D graph was established.
• The uncertainty interval (L, U) was compared with the acceptance limits.

Acceptance Limits ?

Acceptance limits are a threshold value. They are proposed by the result user and expressed using %. As such, acceptance limits are variable according to the requirements of the analyst or the objectives of the analytical procedure. For example, ? =1–2% for an analysis of raw materials, 5% for pharmaceutical specialties or 15% for bioanalysis[4].

In this research, the acceptance limit used was ±15% as bioanalytical methods were being tested for validation.

Table 1. Summary of Analytical Methods Used for the Determination of Oxycodone and Imatinib in Human Plasma

Name of analyte	Oxycodone	Imatinib
Matrix	Human EDTA K2 plasma	Human EDTA K2 plasma
Extraction	SLE	PP
Internal standard	Oxycodone-d6	Imatinib-d8
Mass spectrometer and ionization mode	API4000, ESI+	API4000, ESI+
Concentartion range	0,25–250	10–10000
Concentration unit	ng/ml	ng/ml
Validation concentration	0,25; 0,75; 125; 200	10; 30; 5000; 8000
Production concentration	0,75; 10; 125; 200	30; 400; 5000; 8000

ESI+: electrospray ionization in positive mode, SLE: Supported liquide extraction, PP : protein precipitation.

Response Function

The response function was determined using the results obtained after calibration. The response function can be defined as the relationship between the response (signal or material response) and concentration X. The F function that characterizes this relationship is:
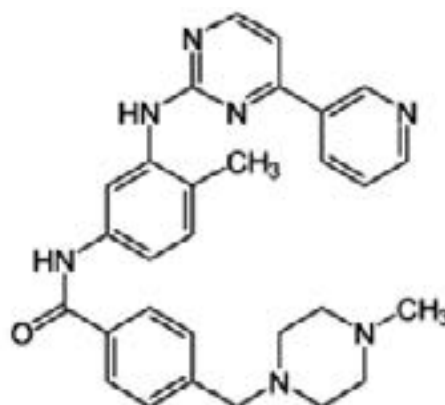
$$Y = F(x) + \varepsilon \qquad ...(1)$$
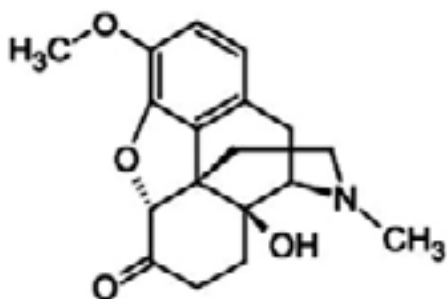
Where e is the residual error, it can be defined as the error associated with the response function F

In this research, the simple linear function was characterized by the following equation:

$$Y = a(x) + b \qquad ...(2)$$

Fig. 1. Oxycodone

Fig. 2. Imatinib

Table 2. Reverse Prediction in the Analysis of Oxycodone in Human Plasma

Concentration (ng/l)	Replicate	Days					
		1	2	3	4	5	6
0,25	1	0,28	0,25	0,26	0,24	0,25	0,24
	2	0,27	0,24	0,26	0,24	0,25	0,23
	3	0,28	0,24	0,25	0,25	0,25	0,23
	4	0,27	0,25	0,24	0,25	0,25	0,23
	5	0,27	0,25	0,24	0,25	0,26	0,23
	6	0,28	0,23	0,25	0,24	0,23	0,23
0,75	1	0,78	0,74	0,71	0,72	0,74	0,70
	2	0,77	0,70	0,72	0,69	0,75	0,70
	3	0,74	0,71	0,74	0,72	0,74	0,70
	4	0,75	0,72	0,71	0,69	0,73	0,73
	5	0,75	0,71	0,71	0,68	0,75	0,74
	6	0,72	0,74	0,73	0,68	0,76	0,73
125	1	121,93	123,53	120,40	119,88	127,08	124,55
	2	123,72	124,47	123,42	121,84	124,93	124,21
	3	122,39	123,97	120,79	119,59	126,30	122,47
	4	121,38	123,43	123,53	119,81	126,94	123,62
	5	121,22	121,56	123,04	119,09	127,56	125,17
	6	123,12	121,55	120,76	121,32	124,90	124,29
200	1	201,91	194,36	199,71	192,84	207,39	197,25
	2	200,98	196,94	196,60	193,57	210,20	197,11
	3	199,92	195,80	198,84	194,21	209,81	197,31
	4	201,45	195,93	200,59	192,85	209,44	197,90
	5	200,45	196,71	198,88	191,97	214,13	196,06
	6	198,65	193,78	199,58	194,11	208,96	199,84

Here, a is the slope and b is the originally ordained[5].

Calculation of Inverse Concentrations (Table 2 and Table 3)

Inverse concentrations or inverse predictions are the feedback concentrations in the response function[5].

In this research, the inverse concentrations for the response function were obtained according to the following formula:

$$x = (y-b)/a \qquad ...(3)$$

Calculation of the Tolerance Interval type ß- ? Content.

Where [L (X), U (X)] is an interval based on the data vector X, X = (X1, …, Xn) is a data sample drawn from a cumulative (continuous) distribution of F using the random variable Z. The interval [L (X), U (X)] is then the tolerance interval for F of ß contained with a confidence γ (or (β, γ) – tolerance interval) if:

$$P (F (U(X))- F (L(X)) \geq \beta) = \gamma \qquad ...(4)$$

Therefore, at least a proportion ß of the population modelled by F is contained in the interval [L, U] with a confidence coefficient of γ [10-12].

In this work, the Liao-Lin-Iyer[11, 12] method, which is based on the Monte Carlo simulation, was used to calculate the tolerance interval through the following algorithm using a number of simulations for K=10000 where i = 1, 2, …, K :

$$\bar{y} = \frac{1}{n}\sum_{i=1}^{p}\sum_{j=1}^{n_i} y_{ij} \; avec \; n = \sum_{i=1}^{p} ni$$

$$SS_b = \sum_{i=1}^{p} n_i (\bar{y_i} - \bar{y}) \; et \; SS_e = \sum_{i=1}^{p}\sum_{j=1}^{n_i}(y_{ij} - \bar{y_i}) \qquad ...(5)$$

The mean squares MSb and MSe were identified as:

Table 3. Reverse Prediction in the Analysis of Imatinib in Human Plasma

Concentration (ng/l)	Replicate	Days				
		1	2	3	4	5
10	1	9,46	10,28	9,92	9,69	8,94
	2	9,64	9,71	11,24	10,55	10,43
	3	9,51	10,39	10,02	10,09	10,37
	4	11,03	10,23	9,99	9,88	9,97
	5	9,71	10,35	10,78	9,35	8,89
	6	12,58	9,90	10,34	10,37	8,69
30	1	31,68	28,56	29,88	29,72	28,23
	2	29,03	30,13	28,67	30,42	30,25
	3	31,67	29,66	30,10	29,99	29,82
	4	31,74	30,43	29,73	30,26	29,97
	5	30,96	30,03	30,57	29,78	29,51
	6	27,19	30,61	28,55	30,39	30,24
5000	1	4559,77	4752,63	4919,21	5046,32	5006,11
	2	5119,55	4815,70	4893,39	5034,08	4914,78
	3	4915,58	4829,49	5001,62	4946,21	5004,03
	4	5904,43	4719,72	4972,73	4874,40	4983,72
	5	4633,30	4744,61	4938,22	5013,00	4998,56
	6	4587,73	4790,18	4881,54	4971,13	4998,40
8000	1	8028,48	7641,16	7909,01	7867,78	7953,80
	2	8610,33	7802,65	7987,19	8093,63	7940,22
	3	7470,81	7796,86	7893,60	7665,54	7922,61
	4	7202,23	7820,88	7958,41	8042,01	7754,34
	5	8405,82	7746,37	7950,00	7804,78	8007,04
	6	8739,63	7784,91	7988,07	7788,05	7791,23

$$MS_b = \frac{SS_b}{(p-1)} \quad et \; MS_e = \frac{SS_e}{(n-p)}$$

...(6)

$$L = \frac{\left(\frac{1}{n_i}\right)\left(1+\frac{1}{p}\right)SS_b}{T_{b,i}^2} + \frac{\left(1-\frac{1}{n_i}\right)SS_e}{T_{e,i}^2}$$

...(7)

With $T_{b,i}^2 \sim \chi_{p-1}^2$ and $T_{e,i}^2 \sim \chi_{n-p}^2$ random numbers generated from the chi-square distribution.

The square root of L multiplied by the quantile of a normal distribution (Z(1+ß)/2) gives the statistical margin of error D used to calculate the ß- content type tolerance interval according to the following formula:

$$U_K = \bar{y} + D \; et \; L_k = \bar{y} - D \qquad ...(8)$$

Here, U_k is the upper limit of the tolerance interval for a concentration level k.

L_k is the lower limit of the tolerance interval for a concentration level k.

Uncertainty Calculation

The measurement uncertainty is a parameter that characterizes the dispersion of values attributed to a measurand (z). Therefore, the purpose of estimating uncertainty is to evaluate the dispersion of measurements made on a sample.

The measurement uncertainty can be calculated by aggregating it with the tolerance interval (Eq.8) according to the following formula [7, 8]:

$$u(z) = (U\_k - L\_k)/2t(v) \qquad ...(9)$$

Here, t(v) is (1+ γ)/2 quantile of the student t-distribution with a degree of freedom calculated according to the following equation:

$$v = \frac{(R+1)^2}{(R+n_i^{-1})^2 / (a-1) + (1-n_i^{-1}) / (an_i)}$$

...(10)

$$R = max\left[0, \left(\frac{1}{n_i}\right) * \left(\left(\frac{MS_b}{MS_e}\right) - 1\right)\right]$$

...(11)

Decision Profile

The decision profile is a graphical decision-making tool recently developed to evaluate the performance of quantitative analytical procedures.
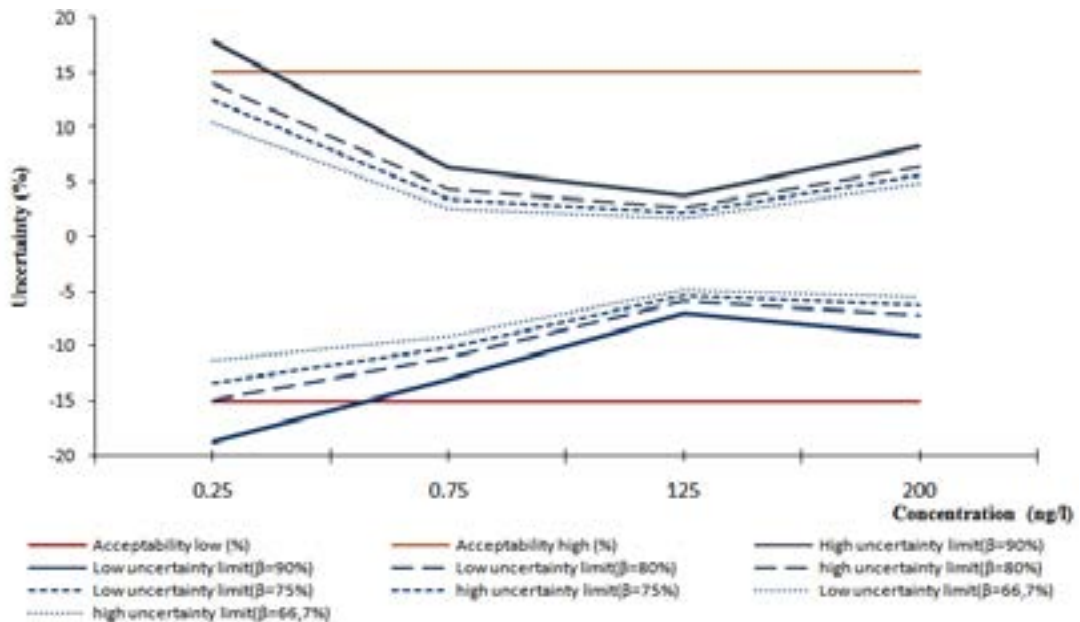

Fig. 3. Decision profiles of the LC-MS method for the determination

The construction of this profile is based on the calculation of uncertainty limits using measurement uncertainty according to the following equation:

Here, k is the coverage factor. The use of the k-factor was based on the desired confidence level. To achieve an approximately 95% confidence level, k=2.

$$\left|\overline{Y} \pm ku_{(z)}\right| < \lambda$$
...(12)

Table 4. Validation results of the LC-MS method for the determination of oxycodone in human plasma by LC-MS using the decision profile (acceptance limit ? = ±15% and ? = 95%)

ß	Conc.	Upper uncertainty limits %	Lower uncertainty limits %	Uncertainty	Expanded Uncertainty	Relative expanded uncertainty	Decision
66,70%	0,25	10,4526	-11,3415	0,014	0,027	10,800	Valid
0,75	2,4872	-9,1539	0,022	0,044	5,813	Valid	
125	1,569	-4,7797	1,984	3,968	3,174	Valid	
200	4,7862	-5,452	5,119	10,238	5,119	Valid	
75%	0,25	12,7379	-13,6268	0,0165	0,033	13,200	Valid
0,75	3,3594	-10,026	0,0251	0,050	6,693	Valid	
125	2,1666	-5,3773	2,3575	4,715	3,772	Valid	
200	5,7499	-6,4158	6,0828	12,166	6,083	Valid	
80%	0,25	14,0726	-14,9615	0,0181	0,036	14,480	Valid
0,75	4,3321	-10,9988	0,0287	0,057	7,653	Valid	
125	2,5883	-5,799	2,621	5,242	4,194	Valid	
200	6,4198	-7,0856	6,7527	13,505	6,753	Valid	
90%	0,25	18,1193	-19,0082	0,0232	0,046	18,560	Invalid
0,75	6,2139	-12,8805	0,0358	0,072	9,547	Valid	
125	3,7966	-7,0073	3,3762	6,752	5,402	Valid	
200	8,4264	-9,0922	8,7593	17,519	8,759	Valid	

Table 5. Validation results of the LC-MS method for the determination of Imatinib in human plasma using LC-MS using the decision profile (acceptance limit ? = ±15% and ? = 95%).

ß	Con.	Upper uncertainty limits %	Lower uncertainty limits %	Uncertainty	Expanded Uncertainty	Relative expanded uncertainty	Decision
66,70%	10	11,7267	-10,1933	0,548	1,096	10,960	Valid
30	4,1646	-4,6602	0,662	1,324	4,413	Valid	
5000	4,7527	-7,7258	155,981	311,961	6,239	Valid	
8000	3,9540	-6,1478	202,035	404,069	5,051	Valid	
75%	10	13,6594	-12,126	0,645	1,289	12,892	Valid
30	4,9699	-5,4654	0,783	1,565	5,217	Valid	
5000	6,0141	-8,9872	187,056	374,112	7,482	Valid	
8000	4,9209	-7,1147	240,713	481,426	6,018	Valid	
80%	10	15,2327	-13,6993	0,723	1,447	14,466	Valid
30	5,628	-6,1235	0,881	1,763	5,876	Valid	
5000	6,9315	-9,9046	210,452	420,903	8,418	Valid	
8000	5,5916	-7,7854	267,542	535,084	6,689	Valid	
85%	10	16,9265	-15,3931	0,808	1,616	16,160	Invalid
30	6,3421	-6,8377	0,989	1,977	6,590	Valid	
5000	7,8730	-10,8462	233,990	467,980	9,360	Valid	
8000	6,4017	-8,5955	299,944	599,888	7,499	Valid	

RESULTS AND INTERPRETATIONS

Table 4 presents all results necessary to validate the decision profile method for determining oxycodone levels in human plasma using LC-MS.

For a confidence value of (γ=95%) and for a value of ß between (66.7% and 80%), in all concentration levels, the uncertainty limits do not exceed the acceptance limits set at ±15%. In addition, the relative expanded uncertainties
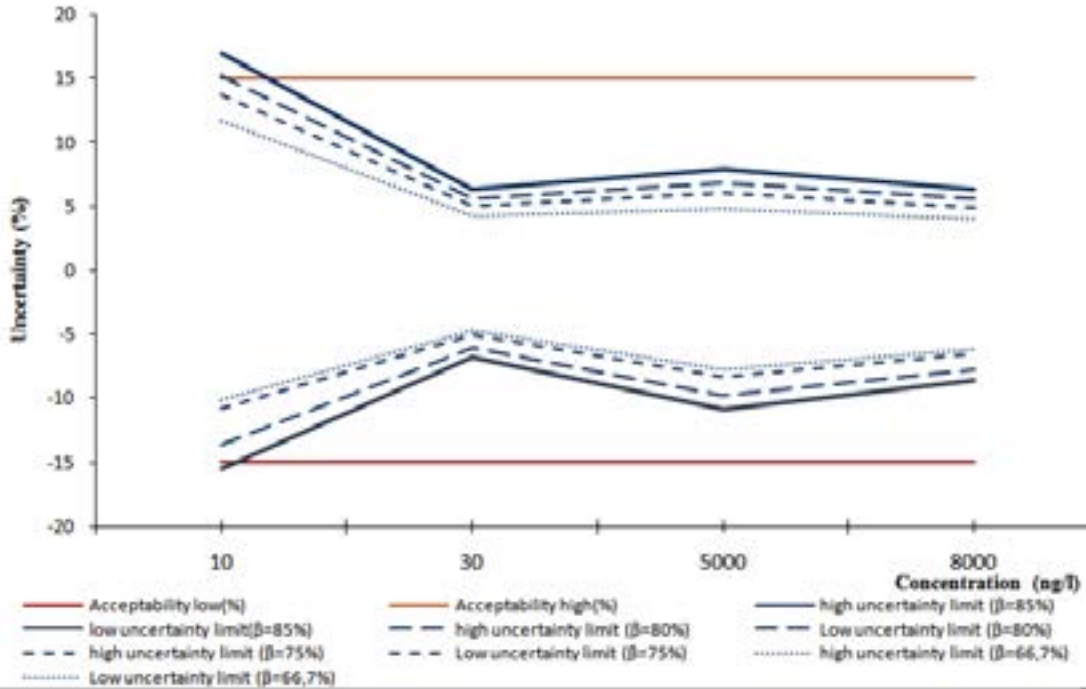

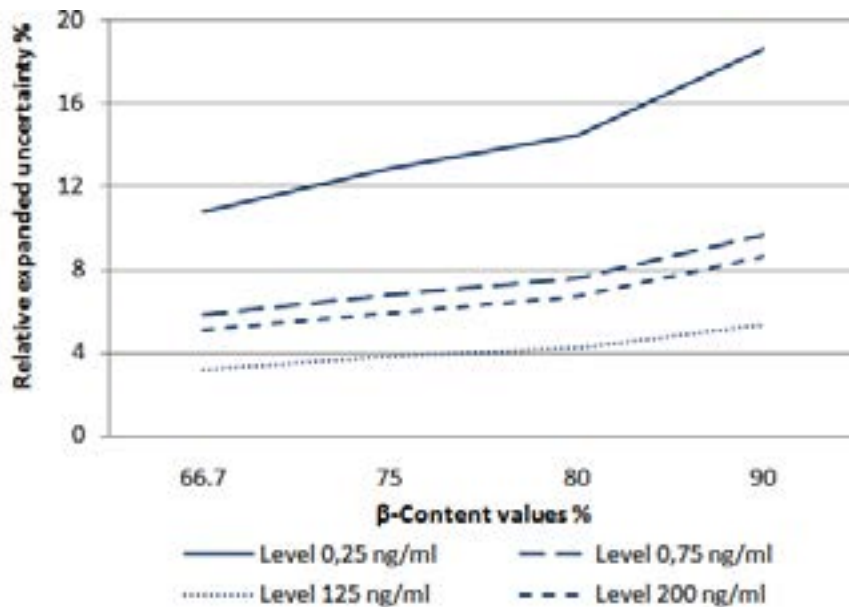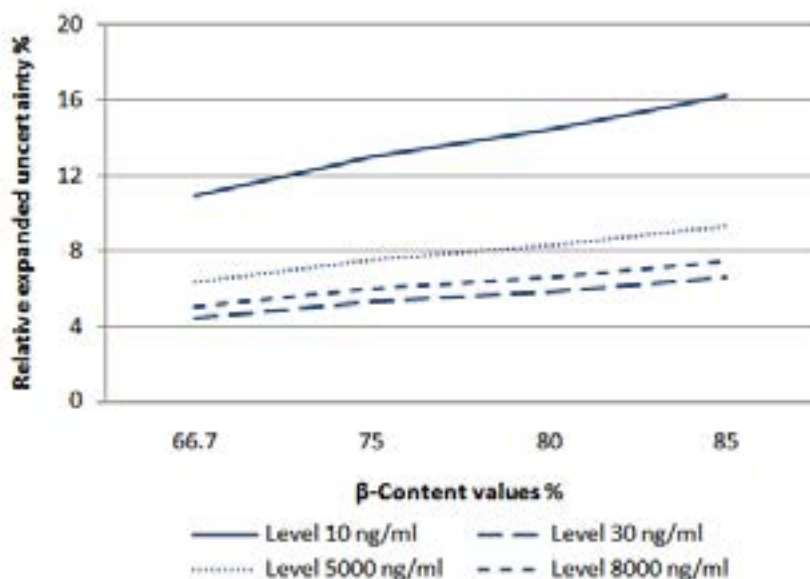**Fig. 4.** LC-MS decision profiles for the determination of Imatinib

Fig. 5. Proportionality between the relative expanded uncertainty

Fig. 6. Proportionality between the relative expanded uncertainty

(%) obtained by dividing the corresponding uncertainties with the introduced concentrations do not exceed 15%. Therefore, it can be concluded that the method is valid for the dosing interval under consideration.

For a confidence value of (γ=95%) and for a value of ß= 90%, in concentrations between 0.75 and 200 (ng/l), the uncertainty limits do not exceed the acceptance limits set at ±15%. In addition, the expanded relative uncertainties (%) do not exceed 15%. However, for a concentration of 0.25(ng/l), the uncertainty limits exceed the acceptance limits and the expanded relative uncertainty greater than 15%. In this case, therefore, the method is valid for concentrations between 0.75 and 200 (ng/l) and invalid for a concentration of 0.25(ng/l).

To validate the method for determining imatinib levels in human plasma using LC-MS, the results presented in Table 5 and the decision profile shown in Figure 4 were obtained.

As can be seen for a confidence value of (?=95%), for a value of ß=66.7% and for a value of ß=75%, all uncertainty limits were within the acceptance limits set at ±15% for all concentration levels. In addition, the expanded relative uncertainties did not exceed 15%. Therefore, the method can be considered valid over the dosing interval under consideration.

When γ=95% and ß=80%, for a concentration of 10(ng/l), the upper uncertainty limit slightly exceeded the acceptance limit. This contrasts to the lower uncertainty limit which did not exceed the acceptance limit. The expanded relative uncertainty did not exceed 15%. As such, this method can be considered valid for all concentration levels under consideration.

When γ=95% and ß= 85%, for concentration levels between 30 and 8000 (ng/l), the uncertainty limits did not exceed the acceptance limits. In addition, the expanded relative uncertainties were less than 15%. For the concentration level of 10(ng/l), the acceptance limits were exceeded. In addition, the expanded relative uncertainty was greater than 15%. This method is, therefore, valid for concentration levels between 30 and 8000(ng/l) and invalid for the concentration level of 10(ng/l).

Figure 5, and Figure 6 respectively illustrate the proportionality between the expanded relative uncertainty and the value of ß-content for the validation of the method for the analysis of oxycodone in human plasma and the method for the analysis of imatinib in human plasma by LC/MS, for a confidence value of γ=95%.

These two figures show that the higher the confidence value γ, the higher the expanded

relative uncertainty value. This provides analysts with result credibility obtained using the routine method.

CONCLUSION

These results demonstrate that the decision profile approach is reliable and can be easily used to validate the LC-MS method for determining levels of oxycodone and imatinib in human plasma. This approach, without any additional experiments, combines two essential criteria to produce quality results. These results are analytical validation and the estimation of measurement uncertainty. As such, this gives analysists a greater degree of control over the risks of routinely using the analytical method. It also provides analysists with more complete information on the performance of this method.

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