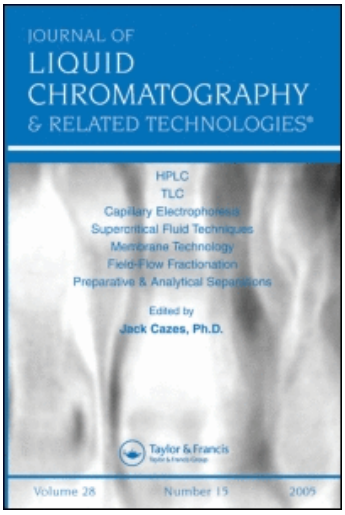

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VALIDATION OF INTERNAL NORMALIZATION FOR IMPURITY ASSAYS

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ABSTRACT

The simplicity of the internal normalization made it a very attractive method. Yet, because of its restrictive applicability requirements, internal normalization is not widely implemented in HPLC quantitative analysis. Basically, applicability requirements are that all the solutes must not only be eluted and detected but must also present similar behavior toward the detection system. Ideally, response factors should be identical for all the solutes or, in practice, of the same order of magnitude. The methodology developed to validate, in a rigorous way, internal normalization was based on the use of a statistical tool called analysis of covariance (ANACOVA). ANACOVA is more or less similar to ANOVA but can manage a continuous variable, like for example, concentration. So, it is possible to use it to compare calibration curves of all the different solutes present in a sample, for example, the main

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product and its impurity. After having checked that for the main product the response factor was the same around the target concentration of the HPLC method, and at low concentration, it was then possible to make comparison with impurity behavior, and to determine whether the use of the response factor was necessary or not. Eventually, ANACOVA enabled the validation of internal normalization by assessing that all the solutes presented required behavior. This methodology was successfully applied to an actual example of liquid chromatography quantitative analysis, taken from the pharmaceutical industry. In this case, internal normalization for impurity assays of an anticytomegalovirus drug substance was validated after response factor correction.

INTRODUCTION

Internal normalization is not as widely used in liquid chromatography as in gas chromatography for quantitative analysis. The reason lies in the strict applicability requirements to allow its implementation. Therefore, its use is restricted to quality control analysis, where the qualitative composition of the sample is known and does not vary. In this perspective, a particular field of interest is the determination of impurity and related substances in pharmaceutical products. In that particular case, internal normalization is a common practice, but preliminarily to its use, a validation is required. After that, its main interest lies in its simplicity and rapidity since no calibration step is henceforth necessary. Effectively in an industrial context, where time saving is a critical concern, a routine quantification method must consist of as few injections as possible.

These advantages induced us to develop a rigorous validation methodology. After a brief review of the internal normalization principle and of its requirements, an approach based on a statistical data handling called ANACOVA is proposed to validate its use. The methodology is then illustrated by an example taken from the pharmaceutical industry. The considered HPLC method is dedicated to the determination of impurity assays of an anticytomegalovirus drug substance. The validation of internal normalization was, in this case, carried out by applying step by step the proposed methodology.

THEORETICAL CONSIDERATIONS

After a short description of the internal normalization method, theoretical bases of ANACOVA are summarized. Then the proposed methodology to validate internal normalization is presented.

Internal Normalization

Principle

The principle of internal normalization, described in depth in literature,[1-5] is rather simple. Provided that for each solute, i, the analytical signal lies within the linearity range, the peak area, A_i, is proportional to m_i, the weight of solute having flown through the detector cell, thus, that was present in the sample injected volume.

$$m_i = K_i A_i \quad (1)$$

K_i : response factor

Therefore, the percentage in weight of each analyte is given by:

$$\%_i = \frac{K_i A_i}{\sum_i K_i A_i} \times 100 \quad (2)$$

Internal normalization presents several advantages. First, this quantification method is really simple to implement in routine, and requires very few injections. Second, it limits mistakes resulting from tedious numerous sample preparations and eliminates errors due to sample weighting and sample injected amount.

Applicability Requirements

-To apply this method in HPLC, several conditions have to be fulfilled:

-All the analytes present in the sample to analyze, and not only solutes of interest, must elute from the column (no irreversible retention), with enough resolution, and furthermore have to be detected.

-Response factors must be either equal, or known, or (at least) experimentally accessible and of the same order of magnitude.

-Calibration curves for all the solutes must be linear and superimposable, taking into account the response factors (absolute or relative).

-The resulting calibration line must present a zero intercept.

The first condition must be established during the method development step and, especially, when specificity is evaluated. In the present paper, it will be considered that these criteria are fulfilled. It means the HPLC method is satisfactory enough and will not be changed unless there are unexpected results. Concerning the second point, as far as we know, no clear approach is commonly

available. So, below is presented the methodology that we have developed to ensure compatibility of response curves for all the solutes. This methodology is based on the statistical tool dedicated to the comparison of regression lines: the Analysis of Covariance, ANACOVA. The third point can be easily tested with a traditional Student's test on the intercept.

ANACOVA: Theory and Use

As ANACOVA is not a common procedure as linear least square regression or one-way ANOVA, its mathematical basis and the usual way to proceed are recalled in this section.[6] Even if ANACOVA computations can easily be achieved with any statistical software, or nearly, a whole theoretical overview including all the formula used presents an undeniable pedagogical interest.

Field of Application

From a statistical point of view, ANACOVA is to be used if the response depends, *a priori*, on both one group variable, values of which are discrete, and a continuous variable. For a given modality of the group variable, variations of the response in the function of the continuous variable are considered linear. The statistical data processing enables the determination, if the modalities of the group variable, slopes, and intercepts are significantly different or not. In the present case, the group variable is the identity of the analyte and the continuous variable is the analyte concentration.

Mathematical Model

The mathematical model is given equation 3.

$$y_{ij\alpha} = a_0 + a_j + b_j x_{ij} + \varepsilon_{ij\alpha} \quad (3)$$

Where $y_{ij\alpha}$ is the result of the α^{th} determination carried out on analyte j at concentration level i, a_0 the estimated value of the average intercept, a_j the estimated value of the deviation from a_0 for the line of analyte j, b_j the estimated value of the slope of the line of analyte j, x_{ij} the value of concentration at level i for analyte j, and $\varepsilon_{ij\alpha}$ the effect of the random error (it is a centered Gaussian variable).

Homogeneity of Variance

Homogeneity of variance between groups is a prerequisite for the use of an unweighted ANACOVA model. So, it is necessary to check for example, through a Cochran's test,[7-8] that the variances of all the groups, *i.e.* the $\sigma^2_{y/x}(j)$: residual variances of the individual linear regressions[9,10] carried out for each group, are homogeneous. If the homogeneity of variances is not rejected, it is possible to calculate a pooled estimate of the residual variance, $\hat{\sigma}^2_p$.

$$\hat{\sigma}^2_p = \frac{\sum_{j=1}^{k} \nu(j)\hat{\sigma}^2_{y/x}(j)}{\sum_{j=1}^{k} \nu(j)} \tag{4}$$

where $\hat{\sigma}^2_{y/x}(j)$ and $\nu(j)$ are, respectively, the estimate of the residual variance of line j and its number of degrees of freedom - the number of degrees of freedom is linked to the number of points of line j by the relation: $\nu(j) = n(j) - 2$ - and k the number of lines. In case the homogeneity of variances is rejected, the use of weighted ANACOVA is required. As it is common use,[9,11] the weights chosen correspond to the inverse of the variance. If the weight of the group j is denoted g_j, then $g_j = \frac{1}{\hat{\sigma}^2_{y/x}(j)}$. Thus, all the weighted residual variances become equal to 1 and also are the weighted pooled residual variance. This way of weighting globally each line, and not each level, by the corresponding regression residual variance is accepTable, since for all the lines, the range of concentration is rather limited. So, the main drawback of weighting, i.e., the low quality of the variance estimates, is overcome. Henceforth, for the sake of simplicity, only the case of unweighted ANACOVA is dealt with. Nevertheless the adaptation to weighted ANACOVA is quite simple, since it only consists in replacing x_{ij} and $y_{ij\alpha}$ by the corresponding weighted values, respectively, $g_j x_{ij}$ and $g_j y_{ij\alpha}$. The same treatment is then applied to all the mean values calculated from x_{ij} and $y_{ij\alpha}$.

Slopes Comparison

If homogeneity of variances has not been rejected, then it is possible to compare slopes of regression lines. Otherwise, it is obviously possible to compare slopes, but only with weighted ANACOVA. The question is: "Are the slopes not significantly different?" and the null hypothesis can be formulated as: "The

slopes of each individual line are equal to the overall mean slope." The estimate of the mean slope, b_c, is given in Equation 5.

$$b_c = \frac{\sum_{j=1}^{k}\sum_{i=1}^{n(j)}\left(x_{ij} - \bar{x}_j\right)\left(y_{ij} - \bar{y}_j\right)}{\sum_{j=1}^{k}\sum_{i=1}^{n(j)}\left(x_{ij} - \bar{x}_j\right)^2} \tag{5}$$

The estimate of the slope of line j, b_j, is given in Equation 6.

$$b_j = \frac{\sum_{i=1}^{n(j)}\left(x_{ij} - \bar{x}_j\right)\left(y_{ij} - \bar{y}_j\right)}{\sum_{i=1}^{n(j)}\left(x_{ij} - \bar{x}_j\right)^2} \tag{6}$$

If the estimate of the slopes standard deviation is denoted $\hat{\sigma}_b^2$, then $\hat{\sigma}_b^2 = \frac{Q_b}{\nu_b}$

Where

$$Q_b = \sum_{j=1}^{k}\left[b_j^2\sum_{i=1}^{n(j)}\left(x_{ij} - \bar{x}_j\right)^2\right] - b_c^2\sum_{j=1}^{k}\sum_{i=1}^{n(j)}\left(x_{ij} - \bar{x}_j\right)^2 \tag{7}$$

and $\nu_b = k - 1$ (8)

The statistical test uses the observed values of the Fischer-Snedecor's test discriminant function $F = \frac{\hat{\sigma}_b^2}{\hat{\sigma}_p^2}$. If the numerical value of F is not greater than the critical value of a Fischer-Snedecor's variable with ν_b and $\nu_p = \sum_{j=1}^{k}\nu(j)$ degrees of freedom at significance level α, then the null hypothesis cannot be rejected. α must be defined, *a priori*, it means before carrying out the test. Usual

values for α are 0.05 or 0.01, which corresponds to a risk of rejecting the null hypothesis while it is valid, respectively 5 times and once out of 100. In practice, if the null hypothesis cannot be rejected, it means that the slopes of all the lines are considered equal, otherwise they are different.

Intercept Comparison

If slope identity has not been rejected, it is possible to compare intercepts of regression lines. In case slopes are different, it is totally meaningless to look for a common intercept since lines are anyhow different. The question is: "Are the intercepts not significantly different?" and the null hypothesis can be formulated as: "The intercepts of each individual line are equal." The estimate of the intercept of line j, a'_j, is given in Equation 9.

$$a'_j = \frac{\sum_{i=1}^{n(j)} y_{ij} - b_c \sum_{i=1}^{n(j)} x_{ij}}{n(j)} \tag{9}$$

If the estimate of the intercepts standard deviation is denoted $\hat{\sigma}_a^2$, then $\hat{\sigma}_a^2 = \frac{Q_a}{\nu_a}$

where $Q_a = \sum_{j=1}^{k} n(j)\left(a'_j - \overline{a'}\right)^2$ (10)

with $\overline{a'} = \frac{1}{\sum_{j=1}^{k} n(j)} \sum_{j=1}^{k} n(j) a'_j$ (11)

and $\nu_a = k - 1$ (12)

The statistical test uses the observed values of the Fischer-Snedecor's test discriminant function $F = \frac{\hat{\sigma}_a^2}{\hat{\sigma}_p^2}$. At this level the pooled estimate of the residual variance, $\hat{\sigma}_p^2$, must take into account the equivalence of the slopes. Therefore, it is calculated according to equation 13 instead of equation 4.

$$\hat{\sigma}_p^2 = \frac{\sum_{j=1}^{k}\sum_{i=1}^{n(j)}\left(y_{ij} - a'_j - b_c x_{ij}\right)^2}{\sum_{j=1}^{k} n(j) - 1 - k} \qquad (13)$$

If the numerical value of F is not greater than the critical value of a Snedecor's variable with v_a and $\nu_p = \sum_{j=1}^{k} n(j) - 1 - k$ degrees of freedom at significance level α, then the null hypothesis cannot be rejected. In practice, if the null hypothesis cannot be rejected, it means that the intercepts of all the lines are considered equal.

Conclusion

Previous theoretical developments have demonstrated how ANACOVA could establish if different regression lines could be considered identical or not. The statistical process included three steps: homogeneity of variances, equality of slopes, equality of intercepts. The order was of much importance. since the results of each step were the determining factor to carry out the next one. Identity of lines was acquired only if all the criteria were met. Henceforth it will be shown how ANACOVA can be used to validate internal normalization for impurity assays.

Methodology to Validate Internal Normalization for Impurity Assays

To validate internal normalization for impurity assays, it must be established that calibration curves for all the solutes are linear, and that regression lines are not significantly different from each other, if need be, taking into account response factors or relative response factors. Many approaches are described in the literature to check linearity.[10,12-16,17] As a consequence, this point will be considered acquired and will not be treated below. Further developments will focus on the methodology used to ensure equivalence of lines for the different solutes.

Main Product Within a Low Concentration Range

From a theoretical point of view, it is obvious that the response factor is unique for a given analyte. Yet in practice, because of experimental troubles,

measured values of the response factor within very different concentration ranges can appear different. So, the use of ANACOVA is proposed to verify that regression lines obtained around the target concentration of the HPLC method, and at low concentration (same order of magnitude as those of impurity), are the same. Obviously in this case, the use of weighted ANACOVA is nearly always necessary. Absolute dispersion on the response cannot be the same when values vary by a factor of 100. Obviously, the use of a correction with a response factor is totally prohibited, since it is the same compound. If regression lines are not the same, further investigations have to be carried out, especially concerning the accuracy of sample preparation and the possibility of matrix effects. This first step can be bypassed to save experimental time, but in case of further discrepancies a doubt can subsist.

Comparison of Calibration Curves

Then, a comparison of the behavior of the main product and of impurity is to be carried out. Again, in this case and for the same reasons as previously, the use of weighted ANACOVA is nearly always necessary. If the results of ANACOVA lead to the conclusion that all regression lines could be considered as being the same, then a Student's test is carried out to check the zero intercept of the common line. Finally, if the zero intercept hypothesis cannot be refused, internal normalization for impurity assays without response factor (it meant they could all be considered equal) is validated. Significant differences between intercepts of regression lines or a non zero intercept for the common line must lead to further investigations, for example about matrix effects, and appropriate corrections. If the results of ANACOVA lead to the conclusion that slopes of regression lines are not identical, a response factor correction is to be considered. The response factors can come either from previous experiments, from literature data, or the relative response factors can be computed from the data of individual regression carried out separately on each compound. Then the correction taking into account these response factors is applied to all the solutes.

If response factors stem from a set of data independent of those used for validation, a whole new ANACOVA, including both slopes and intercepts comparison, is to be carried out to check that slope correction was suitable. If relative response factors are determined from the validation set of data, ANACOVA is useful only to compare intercepts since slopes are equal by construction (a statistical test on slopes would not be false but of no use). In any case, internal normalization for impurity assays with response factor is validated if no significant differences between regression lines are encountered, and if the zero intercept of the single final regression line cannot be refused. The zero intercept test is carried out on the final regression line to maximize the power of the test.

Significant differences between regression lines or a non zero intercept must lead to further investigations and appropriate corrections. The whole validation process corresponding to sections Main Product Within a Low Concentration Range and Comparison of Calibration Curves is illustrated by the scheme given in Figure 1.

EXPERIMENTAL

Our methodology will now be applied to an example taken from the pharmaceutical industry. The product of interest is an anticytomegalovirus drug substance developed by Rhône-Poulenc Rorer and analyzed by HPLC-UV.

Drug Substance

This anticytomegalovirus drug consists of a main product, denoted RPR111423, and of eight impurities denoted a, b, …h. For confidentiality reasons, it was not authorized to give in the present paper, the structure of the impurity. Characteristics and formula of the main compound are given in Table 1.

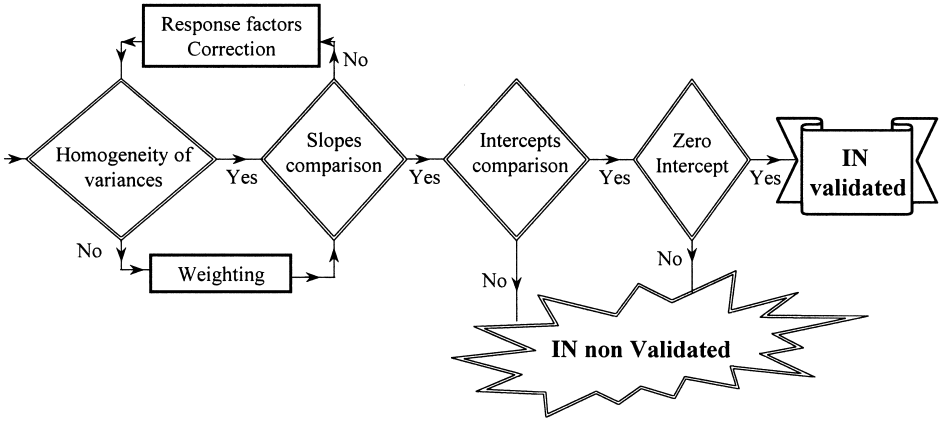
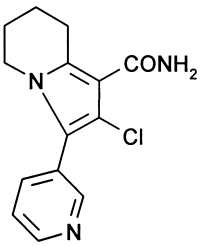


Figure 1. Most general ANACOVA process used to validate Internal Normalization (IN).

Table 1. Characteristics of the Main Product

Name	Formula	Molecular Weight	Chemical Structure
RPR111423	C14H14ClN3O	275.74	

HPLC Method

The HPLC method used gradient elution reversed phase chromatography. A phosphate buffer solution was prepared by dissolving 4.1 g of potassium dihydrogenophosphate (KH_2PO_4) and 5.2 g of dipotassium hydrogenophosphate (K_2HPO_4), both analysis grade, in 2 L of water. The pH was then adjusted to 7.0 by means of a potassium hydroxide solution (KOH) 1 mol/L. Mobile phase A was a mixture of buffer solution, acetonitrile, and methanol 70:20:10 (v/v/v). Mobile phase B was a mixture of buffer solution, acetonitrile, and methanol 55:35:10 (v/v/v). Conditions of the gradient are given in Table 2.

The column used was a Waters Symmetry® C_{18}, pore diameter: 100 Å, particle size: 3.5 µm, length 150 mm, internal diameter 4.6 mm. The pump was a quaternary Hewlett Packard 1100 and the flow rate was set at 1 mL/min. Under these conditions, the back pressure was around 180 bars. The automatic injector was a Hewlett Packard 1100, the injected volume was 20 µL, and samples were at room temperature.

The detector used was a Hewlett Packard 1100. The detection wavelength was set at 230 nm. The column temperature was maintained at 30°C by means of a Hewlett Packard 1100 oven. Resulting chromatograms were produced and processed by Hewlett Packard Chemstation acquisition software. Under these conditions, the analysis lasted 30 min.

The content of the injected samples was expressed as a percentage of the target concentration of the method, which was 140 mg/L. A typical chromatogram is given in Figure 2.

Table 2. Gradient Used

Time (min)	%A	% B
0	100	0
13	100	0
18	0	95
25	0	95
25.1	100	0
30	100	0

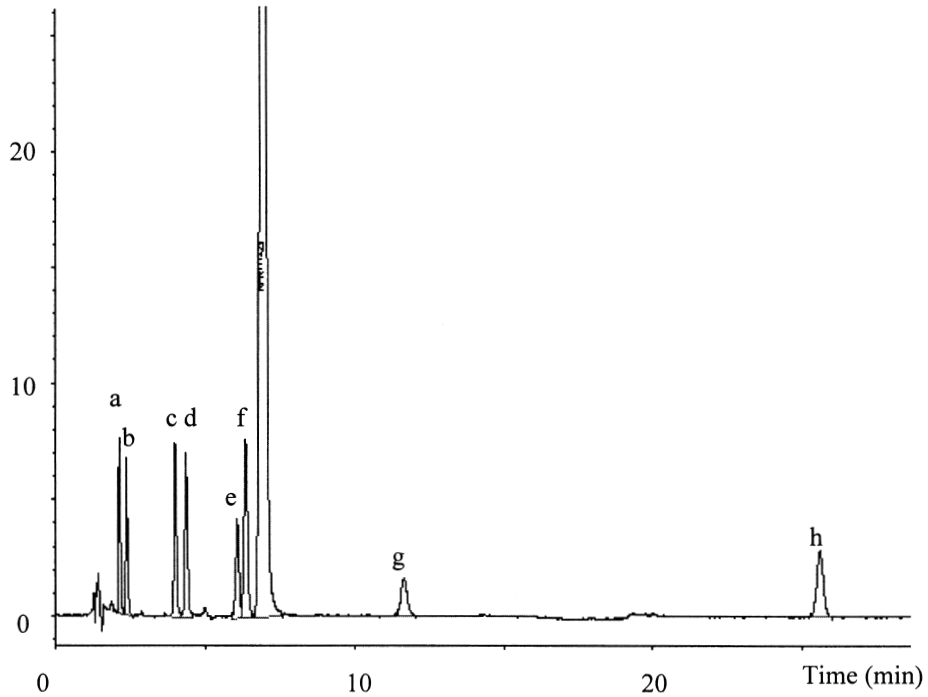


Figure 2. Chromatogram of the main product spiked with all the impurities.

Experimental Design

Several ranges of concentration were prepared, both for the main product and impurity, by dilution from stock solutions at respectively 560 mg/L for the RPR111423 and 140 mg/L for the impurity. The dilution solution used was pure water with 1% of methanesulfonic acid (CH_3SO_3H). Dilutions were carried out with a burette and volumetric flasks.[18] Concerning the main product, a range of concentration around the target concentration of the method (140 mg/L = 100%) was considered first. Samples were prepared at 80%, 90%, 100%, 110%, 120%, 130%, and 140%, and each sample was injected three times. Again for the main product, a second range, but at low concentration, was considered. Samples were prepared at 0.05%, 0.1%, 0.25%, 0.5%, 1%, 2.5%, 5%, 10%, 15%, and 20%, and each sample was injected twice. Finally, for each impurity, a range at low concentration was considered. Samples at 0.05%, 0.1%, 0.5%, 1%, 5%, and 10% were prepared and each sample was injected once.

RESULTS AND DISCUSSION

For both the main product and the impurity, the response chosen was the area of the corresponding chromatographic peak. Statistical calculations were carried out using JMP®[19] (while graphics were processed using Microsoft Excel®). All the statistical tests were carried out with an α value set to 0.05.

Individual Regression Lines

The first step of data processing consisted in determining, individually, for all the solutes, the characteristics of the calibration line. Standard least square regression was used. At this step, the main product around the target concentration of the HPLC method and at low level were considered as two different sets of data. The characteristics of regression for all the series are gathered in Table 3.

Main Product Within a Low Concentration Range

The uniqueness of the experimental response factor, measured within two ranges at very different concentration, was checked. First, the homogeneity of variance of the two regression lines must be compared. The observed value of the Cochran's test discriminant function was $\frac{23.22^2}{23.22^2 + 6.43^2} = 0.929$. Its probability

Table 3. Characteristics of the Individual Regression Lines

Product	RPR111423 (Low)	RPR111423 (Target)	a	b	c	d	e	f	g	h
Slope	87.77	87.91	69.05	65.01	77.14	102.06	86.33	137.09	55.24	97.24
Intercept	0.40	−16.48	0.55	0.26	10.89	0.45	−0.47	0.25	−0.31	−0.02
Residual standard deviation	6.43	23.22	1.29	0.53	28.93	0.83	1.01	2.51	1.10	0.52
Number of points	20	21	6	6	6	6	6	6	6	6

of occurrence is lower than the critical threshold of 0.05. As a consequence, the hypothesis of homogeneity of regression residual variances was rejected. Weighted ANACOVA was to be used. Weights used for each of both groups were the inverse of the regression residual variance, so numerical values

were $g_{low} = \frac{1}{6,43^2} = 2.42 \cdot 10^{-2}$ and $g_{\text{target}} = \frac{1}{23.22^2} = 1.85 \cdot 10^{-3}$.

Entering an ANACOVA model in a statistical software like JMP is generally easy. The way to proceed is exactly the same as for a factorial two-way ANOVA model. Two factors here, "range" and "level" and their interaction, were entered. But, if for ANOVA both factors were discrete, for ANACOVA one was discrete, "range" that could be *low* or *target*, and the other, "level," was continuous. The equality of the slopes hypothesis corresponded to the non influence of the "range/level" interaction. The sum of squares of the interaction calculated by JMP with the weighted ANACOVA model was Q_b=0.203. Only two groups were considered so v_b=1. Because of the weights used, $\hat{\sigma}_p^2 = 1$. So, the observed value of the discriminant function was $F = \frac{\hat{\sigma}_b^2}{\hat{\sigma}_p^2} = 0.203$. This value was less than 1, so, since an unilateral F test was considered, it could be inferred without computing any probability, that the hypothesis of equality of the slopes could not be rejected. In other words, slopes were not significantly different.

Since slopes were the same, *i.e.*, the interaction was not significant, it was then possible to compare intercepts. The comparison was carried out with a weighted ANACOVA model without interaction. The equivalence of the intercepts corresponded to the influence of the discrete factor, *i.e.*, the "range" factor. The sum of squares of the "range" factor calculated by JMP with the weighted

ANACOVA model without interaction was Q_a=0.154. Only two groups were considered so v_a=1. Because of the weights used, $\hat{\sigma}_p^2$=1. So the numerical value of the discriminant function was $F = \frac{\hat{\sigma}_a^2}{\hat{\sigma}_p^2} = 0.154$. Here again, the numerical value was less than 1 so the hypothesis of equality of the intercepts could not be rejected. In other words, intercepts were not significantly different.

Slopes and intercepts were not significantly different, so the lines could be considered as being the same. Characteristics of the common single line, obtained from a weighted linear regression, are given in Table 4. From a physicochemical point of view, it was rather comforting to get such a result, since it must not be forgotten that both lines concerned the same product only at different concentration ranges.

At this stage, it was comforting to note that the usual Student's test on the intercept[6,9,16] (observed t Ratio = 0.23 and Prob>|t| = 0.818) showed a zero intercept for the line. It is worthy to note, that the check for the uniqueness of the response factor can also be regarded as a kind of suitability test for a validated method.

Comparison of Calibration Curves

Processes used to handle the problem are similar to those used in the previous section. Only numerical values and conclusions are, henceforth, presented for the sake of conciseness.

First the homogeneity of the variances was evaluated only for impurity. The observed value of the discriminant function of the Cochran's test was

$$\frac{28.93^2}{1.29^2 + 0.53^2 + 28.93^2 + 0.83^2 + 1.01^2 + 2.51^2 + 1.10^2 + 0.52^2} = 0.9865$$

This value was greater than 0.3910, the critical value that a Cochran's variable with 8 variances and 4 degrees of freedom has 95 chances out of 100 not to

Table 4. Characteristics of the Regression Line of the RPR111423

Product	RPR111423
Slope	87.77
Intercept	0.35
Residual standard deviation	1
Number of points	41

exceed. As a consequence, the hypothesis of homogeneity of regression residual variances was rejected. Weighted ANACOVA was to be used. Weights are gathered in Table 5.

Second, slopes were compared. The sum of squares of the interaction calculated by JMP was $Q_b = 298609$. Nine groups were considered so $v_b = 8$. $\hat{\sigma}_p^2 = 1$. The numerical value of the discriminant function was $F = \frac{\hat{\sigma}_b^2}{\hat{\sigma}_p^2} = 37326$. The probability that a Fischer-Snedecor's variable with 8 and 71 degrees of freedom took a value greater than 37326 was less than 1 chance out of 10000, which was less than 5 out of 100, the chosen α value. This way to proceed was strictly equivalent to the comparison of the observed F-value to the limit F-value for $\alpha = 0.05$. Consequently, the hypothesis of equality of the slopes was rejected. As slopes of all the analytes were significantly different, a response factor correction was to be applied to validate internal normalization.

No previous studies had been carried out to estimate relative response factors of impurity in reference with the main product. So, they had to be estimated from the validation set of experimental results. For each factor, the relative response factor was defined as the ratio of the slope of its line to the slope of the main product line. Relative response factors for all the analytes are given in Table 6.

First of all, it can be noticed that all the relative response factors were close to 1. For each compound, the response was divided by the corresponding relative response factor. By determining relative response factors in this way, the equality of the slopes was acquired by construction. So, a similar process as in the former section was then applied to the data only to compare intercepts. Concerning homogeneity of variances for impurity, the observed value of the discriminant function was 0.990. This value was greater than 0.3910, the critical value that a Cochran's variable with 8 variances and 4 degrees of freedom had 95 chances out of 100 not to exceed. As a consequence, the hypothesis of homogeneity of regression residual variances was rejected. Weighted ANACOVA was to be used. Intercepts comparison was carried out with a weighted ANACOVA model with-

Table 5. Weights Used for ANACOVA, RPR111423 and Impurity Without Response Factor Correction

Product	RPR111423	a	b	c	d	e	f	g	h
$g_j = \frac{1}{\hat{\sigma}^2_{y/x}(j)}$	$3.49\ 10^{-3}$	$5.99\ 10^{-1}$	3.50	$1.20\ 10^{-3}$	1.46	$9.83\ 10^{-1}$	$1.59\ 10^{-1}$	$8.29\ 10^{-1}$	3.68

Table 6. Relative Response Factor Coefficients for the Analytes

Product	RPR111423	a	b	c	d	e	f	g	h
f_i	1	0.79	0.74	0.88	1.16	0.98	1.56	0.63	1.11

out interaction. The sum of squares of the "range" factor calculated by JMP was $Q_a = 6.33$. Nine groups were considered so $v_a = 8$. $\hat{\sigma}_p^2 = 1$. The numerical value of the discriminant function was $F = \frac{\hat{\sigma}_a^2}{\hat{\sigma}_p^2} = 0.880$. As this numerical value was less than 1, the hypothesis of equality of the intercepts was not rejected. In other words, intercepts were not significantly different. Finally, a zero intercept test was carried out on the common line values. The observed value of the Student's discriminant function was 0.78. The probability that a Student's variable with 87 degrees of freedom took a value greater than 0.78 was 43.7 chances out of 100. Consequently, the hypothesis of zero intercept could not be rejected.

As slopes and intercepts were not significantly different, the lines could be considered as being the same. The common line exhibited a zero intercept. As a consequence, internal normalization for impurity assays of the RPR111423, taking into account response factors, was validated.

CONCLUSION

Because of its simplicity, internal normalization is a convenient method for HPLC quality control, for example in pharmaceutical analyses. We have presented, in the present paper, a rigorous methodology to achieve validation of internal normalization in chromatographic analysis. A special focus was given to the method for carrying out comparison of the calibration curves for all the solutes. This methodology was based on the use of a rather little known, but nevertheless useful, statistical tool designed, among other things, to compare regression lines: the analysis of covariance. In practice, our approach consisted of three steps: behavior of the main product at low and target concentration, comparison of raw responses for main product and impurity, and if necessary, comparison of responses for main product and impurity after an adapted response factor correction.

Experimental use of this methodology, with an example taken from the pharmaceutical industry, demonstrated its applicability. One can object to the complexity of the calculations involved in such an approach. But similarly to what is now current practice for linear regression, it is really easy to use statistical

software that can achieve quickly the calculations, and requires only a basic statistical knowledge from the user. Besides methodological developments, the present work underlines difficult requirements that have to be fulfilled before being able to use, without any risk of error, internal normalization.

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