ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
13 (1995) 959-970

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Daily validation procedure of chromatographic assay using gaussoexponential modelling

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Received for review 20 July 1994; revised manuscript received 17 November 1994

Abstract

High performance liquid chromatography is one of the most successful analytical methods used for the quantitative determination of drugs in biological samples. However, this method is marked by a lack of performance reproducibility: chromatographic peaks become wider and even asymmetrical as the column ages. These progressive changes in the chromatographic parameters have to be taken into account when evaluating the validation criteria for the method. These criteria change with the ageing process of the column leading to the need for new estimations to assure the quality of the results. Procedures are proposed for the daily determination of some validation criteria using the exponentially modified Gaussian (EMG) model of the chromatographic peak. This modelling has been studied on simulated chromatographic peaks in order to obtain the relationships between chromatographic measurements and EMG parameters.

Keywords: Column ageing; Exponentially modified Gaussian modelling; High performance liquid chromatography; Pharmacokinetic studies; Test procedures; Validation criteria

1. Introduction

The aim of method validation procedures is to quantify the quality of analytical results and to gaurantee the accuracy and precision of subsequent interpretations. Several national and international organisations have defined validation criteria and have proposed adequate test procedures. These tests are often carried out before using a method for the first time: their conclusions can be assumed as valid only as long as the characteristics of the method remain constant. This is, however, not true for chromatographic assays owing to column ageing. Tests for ruggedness can be used to define variation limits for different operating factors, but these are long, expensive and do not explain the change of method characteristics with time.

We propose a procedure which can determine validation criteria on a daily basis, assuring good quality of results during routine use of the method. This procedure uses an exponentially modified Gaussian (EMG) peak representation which is able to approximate and quantify peak asymmetry.

2. Theoretical

2.1. Column ageing

Column ageing is a general phenomenon in all liquid chromatographic methods: Wilson and Fogarty [1] studied an ion-pairing reversed-phase chromatographic assay of bitolterol on end-capped C18 columns, and

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SSDI 0731-7085(95)01328-8

showed a linear increase of capacity factors (half a unit per year) with a linear decrease of plate numbers (400–600 per year). Glajch et al. [2] found a linear decrease in the capacity factor of tryptophan (10% for 100 column volumes) on a C18 column with an acidic solvent. Claessens at al. [3] showed an increase in silanol interactions on C8 and C18 columns which had been used with aqueous buffers of various pH values and ionic strengths mixed with different organic modifiers.

Modification of column characteristics with time results from exposure to agressive factors, such as aqueous mobile phases with high pH, which can dissolve silica supports and hydrolyse the hydrophobic ligands [4–6]. The characteristics of the stationary phase in terms of the pH of the original silica [8–10], length of the ligands [4–6,8] and structure of the siloxane bonds [3,6,8,9] determine its sensitivity to the aggressive factors. The dissolution of the silica produces a void volume at the column head which may alter the peak symmetry and increase the dead volume, thus affecting the k' values. The hydrolysis of ligands leads to a less hydrophobic phase with faster elution of apolar products. A higher degree of products, however, may be retained because of silanol interactions that also lead to peak tailing [8,11,13]. Another factor in ageing is the deposition of insoluble inorganic and organic substances inside the column, decreasing the pore volume or even plugging the column. The peaks are broadened and peak splitting may occur as a result of channelling [11,12].

These observations are in agreement with the non-reproducibility of chromatographic assays from day to day with changes in retention times and peak profiles. As a consequence, it is really necessary to measure peak characteristics every day to verify method validation. Representing chromatographic peaks by a simple Gaussian model does not take into consideration peak asymmetry, and may lead to major errors when wrongly used to estimate column efficiency, with an overestimation of more than 50% when peak asymmetry, measured at 10%, exceeds 1.5 [14].

2.2. Gaussoexponential peak modelling

Gaussoexponential peak modelling has been used by several authors to account for peak asymmetry [15–22]. The convolution product of a Gaussian function with an exponential decay, both appropriately normalised, can be shown in the following equation:

$$\mathrm{EMG}(t) = \frac{A}{\tau} \exp\left[0.5\frac{\sigma^2}{\tau^2} - \frac{(t-t_G)}{\tau}\right] \times \int_{-\infty}^{z} \frac{\exp[-x^2/2]}{(2\pi)^{1/2}}\,dx \quad (1)$$

where $z = [(t-t_G)/\sigma] - \sigma/\tau$, A = peak area, t_G = mean retention time of Gaussian function, σ = standard deviation of Gaussian function and τ = time constant of exponential decay.

The gaussoexponential peak maximum is situated on the descending flank of the primary Gaussian, and the distance detween the Gaussian peak maximum retention time and the centre of gravity is equal to τ (Fig. 1). The four gaussoexponential parameters, A, t_G, σ and τ, are related to the different statistical moments of the peak [15,19], but cannot be directly measured on the chromatograms; Foley and Dorsey [15] and later Anderson and Walters [18] proposed mathematical equations to calculate these parameters from peak width and asymmetry, but their equations have to be used in a limited asymmetry domain to maintain sufficient accuracy. Carr and co-workers [22,23] plotted universal curves allowing graphic determination of gaussoexponential parameters. Because of the poor precision in abaque readings, we propose a different procedure also based on universal ratios, but using tabulated values as described in the experimental section.

2.3. Assay validation procedure

Method validation procedures include statistical and functional criteria as described by different organisations, principally the Food and Drug Administration, the European Economic Community, the International Standardisation Organisation and the International Conference on Harmonisation [24–34]. The statistical criteria are detection and quantification limits, linearity, precision and accuracy; the functional ones are selectivity-specificity and sensitivity.

Limits of detection or quantification

The detecton limit (LD) is the smallest amount of a compound that can be detected in a sample, whereas the quantification limit (LQ) is the smallest amount that can be quantified with given precision and accuracy. These limits

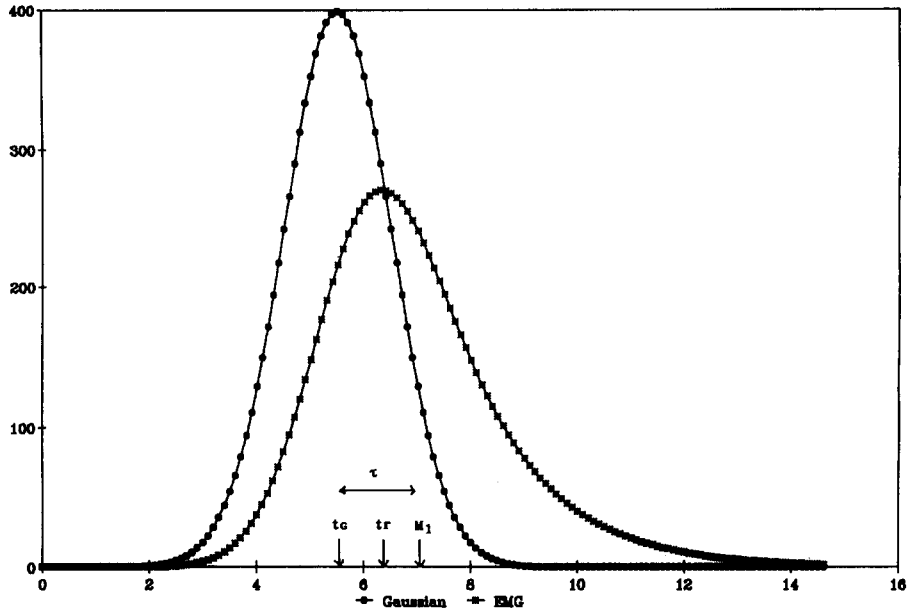

Fig. 1. Comparison of the Gaussian and EMG profiles. The Gaussian function ($t_g = 5.5$ min, $\sigma = 1$ min) was modified by an exponential decay ($\tau = 1.5$ min). t_r = retention time of the maximal height of the EMG peak; M_1 = centre of gravity of the EMG peak.

are determined from the probability density function of a blank sample analysis for which the mean signal is essentially zero. For an α-risk (false conclusion of a significant signal) of 0.14%, LD is taken as three times the standard deviation of the blank. This value of LD corresponds to a second probability density function with a β-risk (false conclusion of no difference from the blank) of 50%. A second limit has been defined which corresponds to twice the LD, so that the β-risk is equal to the α-risk: this second limit was considered as an identification limit [35] or limit of guarantee of purity [36]. In a recent paper, IUPAC gives new definitions of these limits: the first limit is called the critical level ($\alpha = 5\%$, $\beta = 50\%$) and the second corresponds to the LD ($\alpha = \beta = 5\%$) [37]. Given these considerations, the critical level refers to the capacity of experimentals and the LD is a performance characteristic of a measurement process. For the limit of quantification, a similar procedure is applied and, to take into account a precision of 10%, this limit is estimated as ten times the standard deviation of the blank.

For chromatographic assays, assuming a Gaussian distributed noise around the baseline which may be disputed [38], the standard deviation of the blank sample can be derived from peak to peak baseline noise (h). LD and LQ are then calculated using the following equation:

$$\text{LD, LQ} = khR \tag{2}$$

where k is a constant term related to statistic risks, and R is a response factor (concentration per signal).

IUPAC suggests an increase in accuracy with the use of all the regression parameters in the following equation [30]:

$$\text{LD, LQ} = k\frac{\{[s_B^2 + s_i^2 + (i/p)^2 s_p^2]\}^{1/2}}{p} \tag{3}$$

where s_B is the blank sample standard deviation, i, s_i are the intercept and its standard deviation respectively, p, s_p are the slope and its standard deviation respectively and k is a constant term, 3 for LD and 10 for LQ.

This procedure is more time-consuming than the previous ones, but has the advantage in that it gives the LQ not only with defined precision but also with an accuracy, both estimated from the regression parameters.

Linearity

Linearity is taken as the concentration range over which the assay gives results directly proportional to concentration. A least-squares regression procedure can be used to test the dependence of the signal upon the concentration and then confirm the linear modelling of this dependence (Figs. 2 and 3) [39–41]. This test requires constant signal variances and nor-

LEAST SQUARES FITTING ESTIMATIONS:

- slope (p) and its standard deviation (s_p):

$$p = \frac{\Sigma xy - (\Sigma x \Sigma y)/n}{\Sigma x^2 - (\Sigma x)^2/n} \quad \text{and} \quad s_p^2 = \frac{\sigma_{Res}^2}{wx^2 - (wx)^2/n}$$

- intercept (i) and its standard deviation (s_i):

$$i = \frac{\Sigma y \Sigma x^2 - \Sigma x \Sigma xy}{n\Sigma x^2 - (\Sigma x)^2} \quad \text{and} \quad s_i^2 = \frac{\sigma_{Res}^2 . \Sigma x^2}{n\Sigma x^2 - (\Sigma x)^2}$$

- correlation factor (r):

$$r = p.[s_x/s_y] = \frac{\Sigma xy - (\Sigma x \Sigma y)/n}{\sqrt{(\Sigma x^2 - (\Sigma x)^2/n).(\Sigma y^2 - (\Sigma y)^2/n)}}$$

WEIGHTED LEAST SQUARES FITTING ESTIMATIONS:

- slope (p) and its standard deviation (s_p):

$$p = \frac{\Sigma wxy - (\Sigma wx \Sigma wy)/\Sigma w}{\Sigma wx^2 - (\Sigma wx)^2/\Sigma w} \quad \text{and} \quad s_p^2 = \frac{\sigma_{Res}^2}{\Sigma wx^2 - (\Sigma wx)^2/\Sigma w}$$

- intercept (i) and its standard deviation (s_i):

$$i = \frac{\Sigma wy \Sigma wx^2 - \Sigma wx \Sigma wxy}{\Sigma w \Sigma wx^2 - (\Sigma wx)^2} \quad \text{and} \quad s_i^2 = \frac{\sigma_{Res}^2 . \Sigma wx^2}{\Sigma w \Sigma wx^2 - [\Sigma wx]^2}$$

- correlation factor (r):

$$r = p.[s_x/s_y] = \frac{\Sigma wxy - [\Sigma wx \Sigma wy]/\Sigma w}{\sqrt{[\Sigma wx^2 - [\Sigma wx]^2/\Sigma w].[\Sigma wy^2 - [\Sigma wy]^2/\Sigma w]}}$$

Y: mean value of y [= T_O/n].
y_m: mean value of y for one determined value of x.
y_{obs}: observed value of y for each x measured.
y_{est}: estimated value for one value of x.
Σx (Σy): sum of the x (or y).
Σx^2 (Σy^2): sum of the squares of x (or y).
Σxy: sum of the products xy.
T_i: sum of y-values for each x-value.
n: number of observations (x,y).
n_i: number of observations (x,y) for each x-value.
k : number of different x-values tested.
w: weight.
Σwx (Σwy) : sum of the weighted x [or y].
Σwx^2 (Σwy^2): sum of the squares of weighted x [or y].
Σwxy : sum of the weighted products xy.

Fig. 2. Equations used to determine the slope, the intercept and the coefficient of correlation of the linear regression using the least-squares procedure, without or with weighting factors.

mally distributed residuals over the whole concentration range. When variances depend on the magnitude of the signal, a weighted least-squares procedure has to be used, the weighting factor at each concentration being inversely proportional to the variance at the concentration in order for weighted residual variances to become constant.

The dependence between the concentration and the signal is studied using an F-test which allows comparison of the slope to zero. The same test has to be used for the weighted procedure, each residual being multiplied by the weighting factor. When dependence is confirmed, the linear adjustment is checked via a second F-test that compares, for each concentration value, the deviation between the estimated and the mean observed signal to the dispersion of the signal observed around their mean. This second F-test does not apply to the weighted procedure, as this procedure is used to improve a linear fitting once the modelling is known to suit the experimental results: consideration of the results leads to adjustments of the estimated regression line to the observed values measured with good precision, and may lead to an increase of the deviation between scattered observed values and the regression line.

Linearity can also be tested by estimating the coefficient of variation of the response factor for the entire concentration range [25].

Precision

Precision is the statistical variation of results around the mean value. When operating parameters are exactly the same for all the repeated analyses, the term precision refers to repeatability (within-run) and when these factors change, precision refers to reproducibility (between-run). Reproducibility supposes an estimation of the precision of the method between different laboratories. As between-run precision often describes the variation of results within one laboratory, the variation factors being time, equipment or operator, a new concept has been introduced for this between-run precision called intermediate precision [34,42]. These different criteria are evaluated as a coefficient of variation (CV), estimated over 5 to 20 analyses for at least three different concentrations in the linear range. The CV values required are defined by the method to be subse-

source of variation	sum of squares	degrees of freedom
total $\Sigma[y_{obs} - Y]^2$	$Q_T = \Sigma y^2 - [\Sigma y]^2/n$	n - 1
slope $\Sigma[y_{est} - Y]^2$	$Q_{Rg} = p^2[\Sigma x^2 - (\Sigma x)^2/n]$	1
residual $\Sigma[y_{obs} - y_{est}]^2$	$Q_{Res} = Q_T - Q_{Rg}$	n - 2
Dependence between x and y: $F[1;n-2] = \frac{Q_{Rg}/1}{Q_{Res}/[n-2]} > F[1;n-2]$ theoretical		
experimental $\Sigma[y_{obs} - y_m]^2$	$Q_{Exp} = \Sigma y^2 - \Sigma T_i^2/\Sigma_i$	n - k
deviation from linearity $\Sigma[y_m - y_{est}]^2$	$Q_{Adj} = Q_{Res} - Q_{Exp}$	k - 2
Linearity of the dependence between x and y: $F[k-2;n-k] = \frac{Q_{Adj}/[k-2]}{Q_{Exp}/[n-k]} < F[k-2;n-k]$ theoretical		

Fig. 3. Equations used for validation of the linearity based on least-squares fitting.

quently used in routine applications. Reproducibility and intermediate precision can be studied with one or multiple factors ANOVA [24].

Accuracy

Accuracy is the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the experimental value. The accuracy should be studied via mean comparison tests for at least three different concentrations in the linear range and expressed as a relative error. One way of testing this criterion is to estimate the correlation between true and estimated values with a correlation line slope of unity and a zero intercept [43,44].

These last two criteria for validation need samples of known concentration, often called "quality-control" samples, for which the Washington Consensus recommends repeated analysis (five to eight times) at three concentrations, one near LQ, one in the midrange and one near the upper limit of the linear range [32]. For a daily estimation of accuracy, these "quality-control" samples should be assayed in duplicate.

Selectivity and specificity

Selectivity is the ability to obtain a result for one substance independently of other compounds present and supposes that the signal intensity attributed to this substance is not due to other substances. In a chromatographic assay, these two terms are expressed as resolution which is calculated using Gaussian equations [45–47]:

$$\mathrm{Rs} = \frac{2(t_{r2} - t_{r1})}{w_2 + w_1} \tag{4a}$$

or

$$\mathrm{Rs} = \frac{1.18(t_{r2} - t_{r1})}{\delta_2 + \delta_1} \tag{4b}$$

where t_r is the retention time of the peak maximum, w is the peak-width at the base, δ is the peak-width at half-peak height, 2 represents the more retained peak, and 1 represents the less retained peak.

A resolution of unity corresonds to touching peaks with only a slight overlap, which is usually regarded as the minimum resolution required for a separation. For a more precise determination, assuming a peak-width at the baseline of six times the standard deviation, the resolution can be estimated using Eq. (4b) with 0.785 instead of 1.18. However, these equations cannot be used for asymmetric peaks.

Sensitivity

Sensitivity is the smallest concentration difference that can be quantified with sufficient precision. This parameter is often wrongly confused with the detection limit. Sensitivity is estimated from the experimental dispersion, a significant difference between two results being as low as the decrease in experimental fluctuations, with the following relation [24]:

$$\text{Sensitivity} = \frac{(t_{\alpha/2} + t_{\beta})\sigma_{\exp} 2^{1/2}}{p} \tag{5}$$

where t are the Student values relating to the degree of freedom of the experimental standard deviation, for a bilateral α-risk and a unilateral β-risk.

All these statistical and functional validation criteria are derived from the characteristics of the chromatographic peaks, but their variation with time owing to peak broadening and increasing asymmetry means that they are not constant. A system suitability test could help in monitoring the measurement process, but the required test procedure may be time-consuming [48]. In a similar way, we studied a new validation concept with a procedure for the daily estimation of validation parameters using EMG modelling of peak profiles.

3. Experimental

3.1. Material and methods

To work out a new validation procedure in detail it is first necessary to establish a procedure to characterise asymmetric peaks. EMG modelling has been retained for this purpose because of its ability to quantify asymmetry with just one parameter easily related to chromatographic measurements. Non-linear regression could have been used to describe asymmetric peaks, but this modelling system needs adequate software and supposes the impractical use of moments theory to characterise the peaks. However, it is noteworthy that the EMG modelling is not suitable for asymmetric peaks with a B/A value of less than unity. In fact, such a case, mainly due to overload effects, is rarely observed, in particular during the ageing process which is the aim of this work.

Table 1
Tabulated values of the three universal ratios estimated for the measurement of asymmetry (B/A) at 10 and 50% of peak height

B/A at 10%	B/A at 50%	τ/σ	$(t-t_G)/\sigma$	w/σ at 10%	w/σ at 50%
1.000	1.000	0	0	4.292	2.355
1.001	1.000	0.1	0.099	4.313	2.366
1.009	1.004	0.2	0.193	4.375	2.398
1.028	1.014	0.3	0.279	4.470	2.444
1.056	1.027	0.4	0.357	4.593	2.499
1.093	1.042	0.5	0.428	4.737	2.559
1.136	1.060	0.6	0.492	4.898	2.622
1.186	1.080	0.7	0.55	5.074	2.688
1.241	1.102	0.8	0.603	5.261	2.755
1.300	1.124	0.9	0.652	4.458	2.823
1.362	1.147	1	0.697	5.663	2.891
1.427	1.171	1.1	0.739	5.875	2.959
1.493	1.195	1.2	0.778	6.092	3.028
1.562	1.221	1.3	0.814	6.312	3.097
1.631	1.247	1.4	0.848	6.536	3.167
1.701	1.273	1.5	0.88	6.763	3.236
1.771	1.298	1.6	0.911	6.991	3.306
1.841	1.325	1.7	0.94	7.220	3.376
1.913	1.352	1.8	0.967	7.451	3.446
1.984	1.380	1.9	0.993	7.683	3.516
2.056	1.409	2	1.017	7.915	3.586
2.127	1.436	2.1	1.041	8.149	3.657
2.198	1.464	2.2	1.064	8.382	3.728
2.270	1.494	2.3	1.085	8.616	3.799
2.341	1.522	2.4	1.106	8.850	3.870
2.412	1.551	2.5	1.126	9.085	3.941
2.483	1.580	2.6	1.145	9.320	4.013
2.554	1.610	2.7	1.163	9.555	4.085
2.625	1.640	2.8	1.181	9.790	4.156
2.696	1.670	2.9	1.198	10.025	4.228
2.766	1.699	3	1.215	10.260	4.300
2.836	1.729	3.1	1.231	10.495	4.372
2.907	1.760	3.2	1.246	10.731	4.445
2.976	1.788	3.3	1.262	10.966	4.517
3.047	1.819	3.4	1.276	11.202	4.589
3.117	1.850	3.5	1.29	11.437	4.662
3.186	1.879	3.6	1.304	11.672	4.734
3.256	1.910	3.7	1.319	11.908	4.807
3.326	1.940	3.8	1.33	12.143	4.879
3.394	1.970	3.9	1.343	12.378	4.952
3.462	1.999	4	1.356	12.612	5.025
3.531	2.030	4.1	1.368	12.849	5.097
3.601	2.061	4.2	1.379	13.084	5.170
3.669	2.090	4.3	1.391	13.319	5.242
3.738	2.121	4.4	1.402	13.554	5.315
3.806	2.151	4.5	1.413	13.790	5.388
3.875	2.182	4.6	1.423	14.025	5.461
3.943	2.212	4.7	1.434	14.260	5.533
4.011	2.242	4.8	1.444	14.495	5.606
4.079	2.272	4.9	1.454	14.730	5.679
4.146	2.302	5	1.464	14.965	5.751

σ = standard deviation of Gaussian function; τ = time constant of exponential decay; t_G = mean retention time of Gaussian function; τ/σ = first universal ratio, a; $(t-t_G)/\sigma$ = second universal ratio, b; w/σ = third universal ratio, c.

The determination of gaussoexponential para-meters involves the calculation of three universal ratios [22,23]:

ratio $a = \tau/\sigma$

ratio $b = (t_r - t_G)/\sigma$

ratio $c = w/\sigma$

To establish the relationships between these universal ratios and the asymmetry intensity measured as the B/A ratio, we have calculated 50 EMG peaks (τ/σ between 0 and 5) with the recurrent computerised procedure initiated by Berthod [49] on a Lotus 123 spreadsheet. The values of the three universal ratios have been tabulated for each τ/σ value (step of 0.1) (Table 1), and for easier use of this modelling, we fit these relations using a Grapher spreadsheet:

—for measurements at half-peak height:

$$\tau/\sigma = -1.03(B/A)^2 + 6.56(B/A) - 5.18 \quad (6)$$

$$(t - t_G)/\sigma = 2.68(B/A)^3 - 12.44(B/A)^2 + 19.90(B/A) - 9.81 \quad (7)$$

$$w/\sigma = -0.56(B/A)^2 + 4.15(B/A) - 1.14. \quad (8)$$

—for measurements at 10% of peak height:

$$\tau/\sigma = 1.44(B/A) - 0.97 \quad (9)$$

$$(t - t_G)/\sigma = 0.04(B/A)^3 - 0.42(B/A)^2 + 1.56(B/A) - 0.78 \quad (10)$$

$$w/\sigma = 3.33(B/A) + 1.09 \quad (11)$$

These numerical relationships are available for τ/σ between 0.5 and 3. In addition, we have obtained an empirical mathematical relation to calculate the peak-width at 1% from the baseline:

$$w_b = \sigma[0.37(\tau/\sigma)^2 + 3.14(\tau/\sigma) + 5.49] \quad (12)$$

The ability of this EMG model to describe asymmetric peaks in real chromatograms has been tested under different experimental conditions: an apolar product (naphthalene) and a polar one (dimethylaniline) have been analysed on three apolar columns, C18 or C8, with methanol–water as the eluent. Chromatographic data acquisition was performed on an IBM-PC and data were inter-preted with a WORKS spreadsheet. After determination of the width and asymmetry of the peaks at 10 or 50% of the peak height and estimation of the corresponding Gaussian or EMG parameters, the theoretical profiles were constructed. To compare these profiles with the experimental ones, we determined the sum of squares (SSQ) of the deviations between the observed and estimated signal values for each time value (five points per second). This SSQ was expressed as a percentage of the maximum height of the peak using the following equation:

$$CV = \frac{\Sigma(y_{obs} - y_{est})^2/n'}{h_{max}} \times 100 \quad (13)$$

where n' is the number of experimental data per peak.

An example of the superposition of experimental and estimated profiles is shown in Fig. 4 for the analysis of naphthalene and dimethylaniline on an aged Hypersil C18 column. The estimated profiles using the EMG model are more accurate, especially when chromatographic measurements are made at 10% of the peak height. Table 2 gives the SSQ obtained for the simulated profiles of the two products after analysis on the three different columns. In all cases, the results obtained from the EMG model are closer to the experimental data than the results obtained from the Gaussian model. For apolar products like naphthalene, asymmetry should result from mixing chamber effects only, which correspond exactly to the EMG model. For basic compounds like dimethylaniline, however, this asymmetry should be increased by silanophilic interactions following a completely different theoretical model. It can still be seen that the EMG model is adequate and can be used successfully in both cases.

The chromatographic data have been combined with regression results in simple mathematical equations for the determination of validation criteria, each criterion being described in the following.

4. Results and discussion

4.1. Limit of detection

When Eq. (2) is used, the LD depends on the baseline noise and the height response factor, even though quantitative determination mostly involves peak areas. As long as the detector response is constant, the response factor changes only with peak height which is a function of area, peak-width and asymmetry:

—for a Gaussian peak:

$$h_{max} = \frac{A_s}{\sigma(2\pi)^{1/2}} = \frac{A_s 2(-2\ln r')^{1/2} r'}{w(2\pi)^{1/2}} \quad (14)$$

—for a gaussoexponential peak:

$$h_{max} = \frac{A_s}{\sigma(2\pi)^{1/2}} \exp\left[-\frac{(t_r - t_G)^2}{2\sigma^2}\right] = \frac{A_s c}{w(2\pi)^{1/2}} \exp(-b^2/2) \quad (15)$$

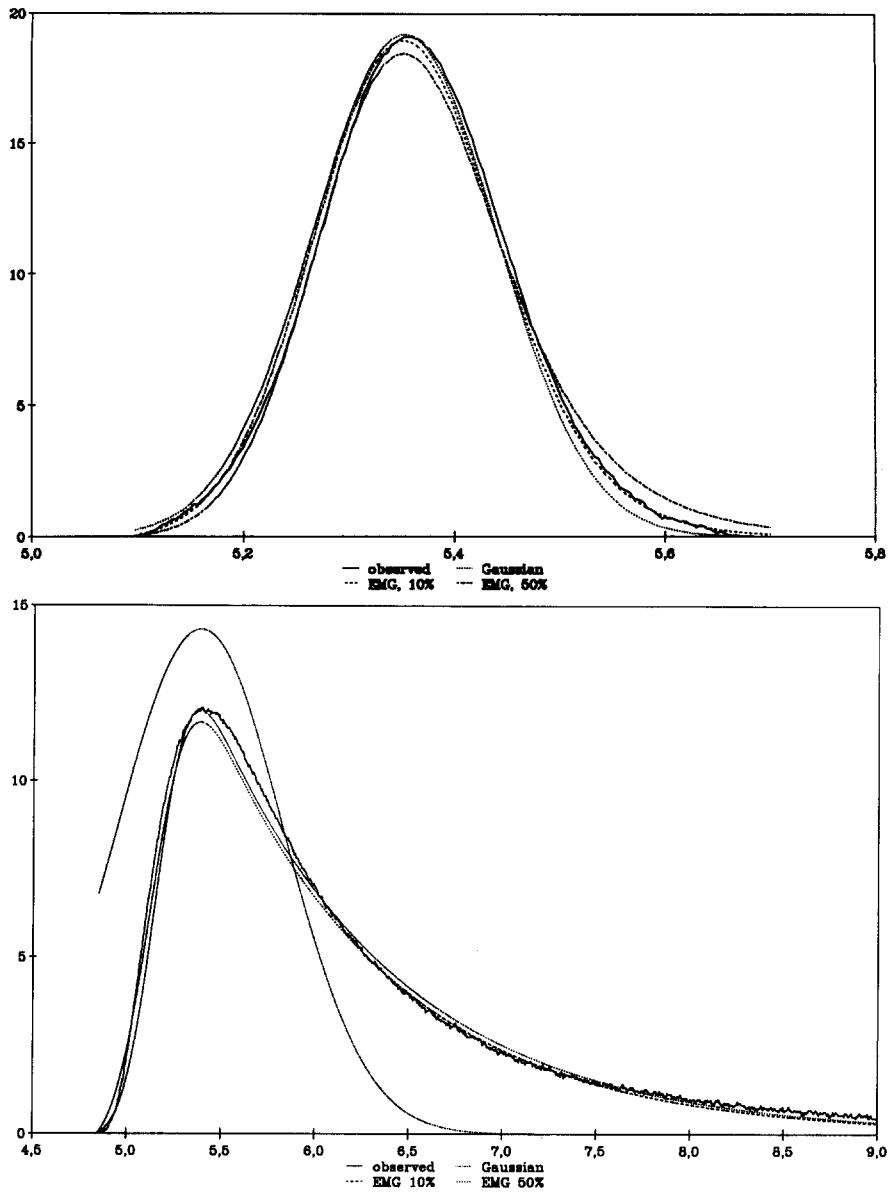

Fig. 4. Comparison of the accuracy of the Gaussian or the exponentially modified Gaussian (EMG) models to fit experimental chromatographic peaks. —— (1) experimental peak; · · · (2) Gaussian fitting; – – – (3) EMG fitting using tabulated values of the universal ratios after determination of the chromatographic characteristics at 10% of the peak height; —·— (4) EMG fitting using tabulated values of the universal ratios after determination of the chromatographic characteristics at 50% of the peak height. (A) Naphthalene; (B) dimethylaniline.

All the constant terms that are independent of the column can be put together in one constant, C_{LD}, defined during the first validation procedure allowing in this way a rapid determination of the detection limit with the following equations:

—for Gaussian peaks:

$$LD = C_{LD} hw \quad C_{LD} = 1.5 C_s \pi^{1/2} / A_s (-\ln r')^{1/2} r' \tag{16}$$

—for gaussoexponential peaks:

$$LD = \frac{C_{LD} hw}{c \exp(-b^2/2)} \qquad C_{LD} = 3 C_s (2\pi)^{1/2} / A_s \tag{17}$$

These rather simple equations avoid standard analysis for daily determination of detection limits, thus eliminating problems with the conservation of standards. They should be very useful for impurity tests which are intended to

reflect the purity of a sample, the main characteristics of these tests being the LD.

4.2. Limit of quantification

For the determination of the limit of quantification using Eq. (3), an estimate of the calibration curve is needed, and the difficulty lies in the calculation of standard deviations. According to Foley and Dorsey [50], the standard deviation of a blank sample can be approximated by one fifth of baseline noise, $h/5$. The standard deviation of the slope is deduced from the correlation coefficient using the following equation:

$$s_p = [p(1-r^2)^{1/2}]/[r(n-2)^{1/2}] \quad (18)$$

The standard deviation of the intercept is obtained from the residual variance:

$$s_i^2 = \frac{\sigma_{res}{}^2 \Sigma x^2}{n\Sigma x^2 - (\Sigma x)^2} = \sigma_{res}^2 f(x)$$

where $f(x)$ is constant when the values of the calibrators used to estimate the regression line remain the same.

Using all these equations, the quantification limit is calculated from the following equation:

$$LQ = \frac{10[(h/5)^2 + \sigma_{res}^2 f(x) + i^2(1-r^2)/r^2(n-2)]^{1/2}}{p} \quad (19)$$

In practice, the intercept is often close to zero with a relative standard deviation of about 100%. Thus, the above equation can be simplified by replacing the intercept standard deviation with the intercept value.

This equation takes into account the baseline noise as well as the regression conditions, thus reflecting every change in the chromatographic system, even imperceptible ones. The daily estimation of the LQ is of principal interest for quantitative purity tests and also for quantatitive measurements of analyte in samples.

4.3. Linearity

The estimation of linearity requires repetitive analyses to assure a sufficiently good estimate of variance over the whole linear range. A good appreciation of the linear fit of calibration data is obtained from the sum of residuals which can be used in the form of a coefficient of variation according to the following formula [48]:

$$CV = \sigma_{res}/(\Sigma y/n) \quad (20)$$

An initial CV value obtained in the first validation procedure can be used as a reference, and comparison with the CV value calculated from daily regression parameters denotes the actual linearity.

4.4. Accuracy and precision

These two other statistical parameters should be tested via quality controls as previously described. Special care should be taken in the preparation of these samples, implementing solutions not already used to establish the calibration curve, and storage conditions have to be defined before method exploitation.

4.5. Selectivity/specificity

As retention times may change from day to day, a blank sample should be studied for all analysis runs and, whenever needed, a sample containing substances known to interact should be prepared for study. The evaluation of these parameters requires determination of resolu-

Table 2
Comparison of the accuracy of the Gaussian or the exponentially modified Gaussian (EMG) models to fit experimental chromatographic peaks

Column	Colosil C18	Ultrabase C8	Hypersil C18
Naphthalene			
B/A 50%	1.38	1.12	1.15
B/A 10%	1.51	1.32	1.13
CV (%)			
Gaussian	8.06	4.03	3.19
EMG, 50%	3.39	1.58	2.86
EMG, 10%	3.02	1.31	2.08
DMA			
B/A 50%	1.20	1.18	2.67
B/A 10%	1.38	1.43	5.72
CV (%)			
Gaussian	5.42	> 5	> 50
EMG, 50%	1.38	2.49	5.67
EMG, 10%	1.56	2.01	4.82

Solutions of naphthalene (25 mg l^{-1}) or dimethylaniline (DMA) (20 mg $^{-1}$) in methanol have been analysed with a water–methanol (25:75 v/v) eluent (1 ml min^{-1}) on three aged columns (150 × 4.6 mm, 5 μm): Colosil C18, Ultrabase C8, Hypersil C18. UV detection at 254 nm.
CV: sum of squares of the residuals expressed as a percentage of the maximal height of the peak. The signal is estimated using the Gaussian model or the EMG model after the determination of the EMG parameters using tabulated values (Table 1) with chromatographic measurements at 10 or 50% of the peak height.

tion. For asymmetric peaks, gaussian equations (Eqs. 4) are not applicable, and analogous equations have to be established after estimation of the baseline peak width on both sides of the retention time. The half-width before the retention time of an EMG peak is equal to the baseline half-width of the primary Gaussian peak plus the difference between the gaussoexponential and primary Gaussian peak retention times. Assuming the width of a Gaussian peak to be six times its standard deviation, the half-peak width before retention time is given by

$$w_- = 3\sigma + (t - t_G) = 3\sigma + b\sigma = \sigma(3 + b) \quad (21)$$

and that after the retention time by

$$w_+ = w - w_- = \sigma[0.37(\tau/\sigma)^2 + 3.14(\tau/\sigma) + 2.49 - b] \quad (22)$$

Thus, the resolution of asymmetric peak can be calculated using the following equation:

$$Rs = \frac{(t_{r2} - t_{r1})}{(w_+)_1 + (w_-)_2} \quad (23)$$

This equation requires determination of the gaussoexponential parameters of individual peaks, which can be obtained at 10 or 50% of peak height, thus allowing the determination of the resolution between two overlapping peaks in a mixture.

Table 3
Statistical and functional criteria of piracetam assay validation measured on three separate occasions

Parameter	New column	Midpoint	End of study
Experimental			
t_r (min)	6.77	6.74	6.67
δ (min)	0.27	0.26	0.27
B/A	1.50	1.60	1.22
h (μV)	200	349	230
SSQ (μV²)	1.41×10^5	4.43×10^5	56.0×10^5
Calculated			
σ (min)	0.071	0.065	0.087
τ (min)	1.165	0.173	0.112
t_G (min)	6.69	6.67	6.60
LD (mg l^{-1})	0.37	0.65	0.43
LQ (mg l^{-1})	1.99	3.88	0.97
CV (%)	0.98	1.74	6.20
Sens. (mg l^{-1})	1.67	2.96	10.2

t_r = retention time; δ, B/A = peak-width and peak asymmetry measured at 50% of the peak height respectively; h = baseline amplitude; SSQ = sum of squares of residuals; LD, LQ = limits of detection and quantification, respectively; CV = coefficient of variation of residual error; sens. = sensitivity.

4.6 Sensitivity

Sensitivity is determined from two variables, slope and experimental standard deviation. Eq. (5) should be used taking into account the residual variance instead of the experimental one, which cannot be determined without replicate analyses.

5. Application

The present approach has been applied to the determination of the drug piracetam from human plasma in a pharmacokinetic study. The calculations have been automated by creating software under DOS. The chromatographic data (peak profile, baseline noise) were calculated by the CR-5A Shimadzu integrator by using BASIC subroutines, with the exception of the asymmetry B/A at half-peak height which was measured manually. The other data supplied to the software were the characteristics of the regression. The software yielded the parameters of the gaussoexponential profile, the limit of detection, the limit of quantification, the CV of the residual error and the sensitivity. This procedure was accomplished on three occasions: at the beginning of the study (on a new column), at the midpoint (after about 200 piracetam determinations) and at the end (after about 400 determinations), as described in Table 3. The retention and the shape of the peak varied only to a minor degree and no sign of column ageing was apparent. Nevertheless, there was some day-to-day variation in the amplitude of the noise level and in the goodness-of-fit (sum of squares of residuals), which resulted in appreciable changes in the limits of detection and quantification, and in the sensitivity. The use of the Gaussian model leads to an LD that is 10–15% smaller, and could lead to erroneous conclusions, especially if the LQ was estimated using Eq. (2).

6. Conclusions

This daily validation procedure allows rapid and simple determination of the main characteristics of a quantitative assay, and provides

better quality assurance for the results than that obtained with a primary validation followed by quality-control analyses. A computer program has been established and tested for pharmocokinetic studies. This procedure enables monitoring of the effect of changes in column characteristics, and in the status of the apparatus (pump, detector) on the performance of the assay. The limit of quantification can be re-evaluated each day, which is of great practical importance in many areas, e.g. in pharmacokinetic studies. The daily measurement of chromatographic parameters helps in the monitoring of retention conditions and column ageing, yielding information which may be valuable for equipment management.

Appendix: list of symbols

a, b, c	universal ratios (see text)
A	peak area
A_s	peak area of the reference concentration used for the determination of the limit of detection
B/A	asymmetry measurement as the ratio of the half-peak widths after and before the maximum retention time
C_s	reference concentration used for the determination of the limit of detection
CV	coefficient of variation
h	peak to peak baseline noise
h_{max}	maximal height of the chromatographic peak
i	intercept of the regression line
IUPAC	International Union of Pure and Applied Chemistry
LD	limit of detection
LQ	limit of quantification
n	number of calibrators used to estimate the regression line
n'	number of experimental data per peak
p	slope of the regression line
r	coefficient of correlation
r'	fraction of the peak height used for measurement of width and asymmetry
R	response factor (concentration per signal)
Rs	resolution between two chromatographic peaks
s_b	standard deviation of signals obtained for a blank sample
s_i	standard deviation of the intercept
s_p	standard deviation of the slope
t	time
t_G	retention time of the centre of gravity
t_r	retention time of the maximum of the peak
t_α	Student value for an α-risk
w	peak-width at baseline
y_{est}	estimated value
y_{obs}	observed value
z	parameter of the EMG function, equal to $(t - t_G)/\sigma - \sigma/\tau$

Greek letters

α	statistical risk of first degree
β	statistical risk of second degree
δ	peak-width at half-peak height
σ_{exp}	experimental standard deviation
σ_{res}	residual standard deviation
σ	standard deviation of a Gaussian peak
τ	time constant of the exponentional decay

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