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System performance and variability of chromatographic techniques used in pharmaceutical quality control

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Abstract

Data representing the routine performance of different chromatographic equipment systems used in pharmaceutical quality control are presented. These performance data allow one to predict long-term relative uncertainties and confidence intervals for different chromatographic procedures and techniques. From the variability that is to be expected, it is apparent that many chromatographic procedures run as routine applications in the pharmaceutical industry are unlikely to be capable of controlling the tight specification limits like those usually demanded for bulk drug substances (active pharmaceutical ingredients), the European 95–105% expectation for finished drugs, or to be used in stability studies. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Chromatographic separation and purification techniques are key elements in the pharmaceutical industry throughout the life cycle of any project and product. Very different techniques and approaches may be used, depending on a project's development.

Neither the Good Laboratory Practice (GLP) or the Good Manufacturing Practice (GMP) guidelines nor the regulatory authorities stipulate which chromatographic techniques and methods are to be used when it comes to quality control for a marketed product. The pharmaceutical manufacturer is free to decide. However, in the case of any conflict the techniques and methods described in a certain product monograph or as a general monograph in the pharmacopoeias have to be used.

This results in an very conservative attitude on the part of quality control as well as regulatory departments when it comes to selecting the chromatographic techniques to be used in product applications and therefore on a long-term for product release and stability testing.

Relying on the remaining standardized and proven technologies (mostly high-performance liquid chromatography, HPLC), however, does not guarantee, that the stringent European expectations, e.g., of a specification limit of 95–105% for assays of finished products or the common 98.0–100.5% for assays of APIs (active pharmaceutical ingredients, bulk drug substances) can be controlled [1–3], or that established system suitability test procedures and the widely proposed acceptance limits (relative standard deviation, RSD≤2%) [4,5] are suited to control the performance that is required for a certain application and that the highly formalized established procedures currently used in case of non-compliance of analytical results with given specification {"out-of-specification (OOS) results" [6–10]} are appropriate to be applied to results derived by using these chromatographic techniques and procedures.

It is a common misconception that the steady

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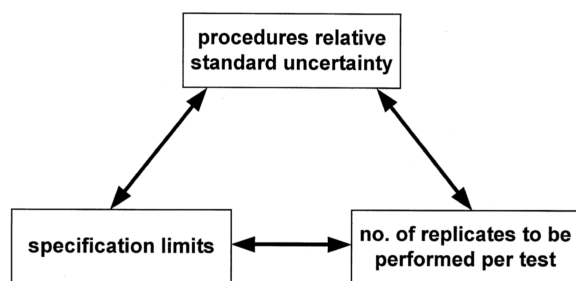


Fig. 1. Interdependency between variability, replicates and specifications.

progress made in chromatographic equipment will automatically lead to "better", i.e., more reliable results. The development of more sophisticated and miniaturized equipment and better separation media may well result in higher sensitivities and higher separation efficacies; however, this will not automatically reduce the analytical uncertainty inherent to any chromatographic procedure [11,12] (see Fig. 1).

This uncertainty has remained unchanged for the last decade, mainly because the chromatographic step is only one of a number of different steps in an analytical procedure (Table 1) that contribute to its overall uncertainty.

Major sources of uncertainty – such as sampling and sample pretreatment – are not even equipment-related. The weakest points in the equipment-related chromatographic steps are still sample injection, transfer and spotting [13,14]. In this work performance data of different chromatographic systems are presented, confirming these uncertainties.

Table 1
Steps of a chromatographic analytical procedure[a]

Step	
1	Sampling
2	Subsampling
3	Weigh in
4	Dissolution
5	Sample clean up/extraction/filtration
6	Concentration
7	Derivatization/complexation
8	Dilution
9	Sample injection / spotting
10	Chromatography
11	Detection
12	Data collection
13	Data conversion
14	Calculation
15	Reporting

[a] For procedures using external standards, this standard usually undergoes steps 3 and 6–15.

2. Experimental

2.1. Apparatus, chromatographic conditions, chemicals and sample preparation

2.1.1. HPLC (robotic system) – system suitability test for HPLC assay of iopamidol

The chromatographic system consisted of a Merck–Hitachi system D 6000 chromatograph (Merck, Darmstadt, Germany) including a Merck UV–Vis detector L-4250 (Merck) set at 254 nm and a Shandon (UK) Hypersil ODS analytical column (250 mm×4.6 mm I.D., 5 μm particle size). The system was integrated into an ISRA (Darmstadt, Germany) robotic system.

The mobile phase, water R (Pharm. Eur.)–methanol (Merck) (94:6, v/v), was delivered at a flow-rate of 1.5 ml/min. The column heater was set at 30°C

and the injection volume was 20 μl (CUT volume technique).

Iopamidol (USP grade) [supplied by Bracco (Milan, Italy)] was dissolved in methanol–water (3:1, v/v) to a concentration of approximately 20 μg/ml.

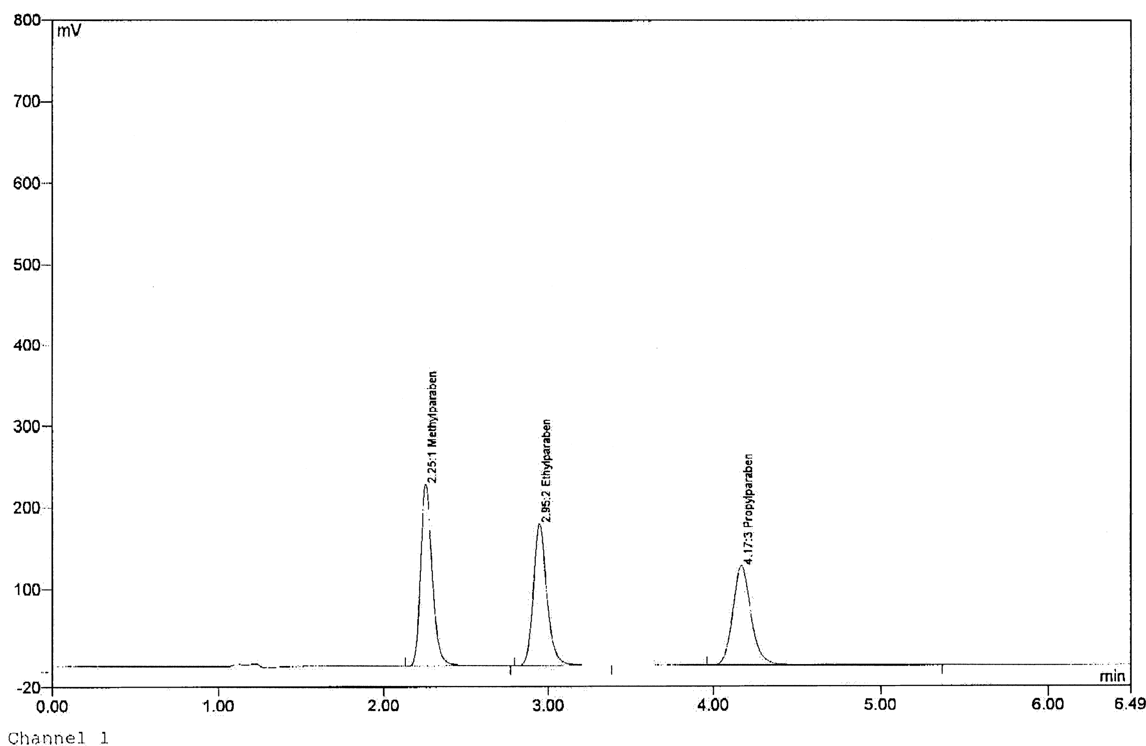
Five replicates were injected and the RSDs of the five injections were calculated from the peak areas.

2.1.2. HPLC – routine system performance verification (Fig. 2)

Merck–Hitachi HPLC systems D 6000 or LaChrom D 7000 or Kontron Kromasystems 2000 (Bio-Tek, Neufahrn, Germany) with UV or diode-array detectors were used.

A stock solution (0.3 mg/ml) of *p*-hydroxybenzoic methyl, ethyl and propyl esters (Sigma–Aldrich, Steinheim, Germany) in acetonitrile was prepared. The stock solution was used to prepare 10, 15, 20 μg/ml standards of *p*-hydroxybenzoic methyl, ethyl and propyl esters in water R (Pharm. Eur.).

Six replicate injections (two injections for each of the three concentrations) of 20 μl of the solutions of *p*-hydroxybenzoic methyl, ethyl and propyl esters in acetonitrile onto a 5 μm Nucleosil (Machery-Nagel, Düren, Germany) C_{18} analytical column (125 mm× 4 mm I.D.) were carried out [column temperature



No	Ret.T min	Type	Name	Area mV*min	Height mV	1/2Width min	Amount
1	2.25	MLR	Methylparaben	17.970	223.01	0.07	10.068
2	2.95	MLR	Ethylparaben	16.709	173.77	0.09	10.276
3	4.17	MLR	Propylparaben	15.650	122.15	0.12	10.640
				50.330	518.93	0.28	30.985

Fig. 2. Original chromatogram of a HPLC system performance verification using a mixture of *p*-hydroxybenzoic acid methyl, ethyl and propyl esters. For details see Experimental.

35°C, mobile phase acetonitrile–water (45:65) at 1 ml/min, UV detection at 254 nm].

RSDs of the six injections (two of each concentration) were calculated from the response factors of the peak areas.

2.1.3. Gas chromatography (GC) direct injection system suitability test for GC assay of menthol

An autosystem gas chromatograph (Perkin-Elmer, Überlingen, Germany) with a flame ionization detection (FID) system and direct injection (autosampler) (Perkin-Elmer) was used.

A packed column (15% OV 101 on Chromosorb, W/AW-DMDCS), 2000 mm×2 mm I.D. (Supelco–Sigma–Aldrich, Steinheim, Germany) with nitrogen (5.0 grade, SWF+Linde, Friedrichshafen, Germany) as a carrier gas (30 ml/min) was used.

Oven temperature was set to 110°C, injector temperature to 240°C and FID temperature to 260°C.

Standard solutions of menthol (Sigma–Aldrich) in methylene chloride (Merck) (concentration 0.4 mg/ml), were prepared.

Routine system suitability tests were performed by six replicate direct injections (1 μl each) from one sample vial containing the relevant reference substance mixture. RSDs of the six injections were calculated from the peak areas.

2.1.4. Headspace GC – system suitability test for GC assay of ethanol

An autosystem gas chromatograph (Perkin-Elmer) with a FID system and a HS 40 headspace autosampler (Perkin-Elmer) was used.

A capillary column (DB-WAX, Fisons Scientific Equipment, Loughborough, UK) 50 000 mm×0.32 mm I.D.; d_f=1 μm with helium (2 ml/min) (SWF+Linde) as a carrier gas was used.

Oven temperature was set to 70°C, injector temperature to 100°C and FID temperature to 200°C.

Headspace conditions were: oven 60°C, equilibration time 30 min, injection time 0.06 min, pressurization time 3.0 min.

Standard solutions of ethanol (Merck) in a concentration of 1 mg/ml in water R (5 ml in a 22-ml headspace vial) were prepared.

Routine system suitability tests were performed by six headspace injections from six vials containing aliquots from one solution off the reference substance. RSDs of the six injections were calculated from the peak areas.

2.1.5. Capillary electrophoresis (CE) – system suitability test for CE assay of 3-methylcamphoric acid

A Hewlett-Packard (Waldbronn, Germany) three-dimensional CE system equipped with a 24.5 cm (capillary inlet to detection window)×50 μm I.D. fused-silica capillary (Hewlett-Packard) was used. The capillary was conducted by rinsing with 1.0 *M* sodium hydroxide for 30 min, then filled with buffer for 5 min and equilibrated for another 3 h with sodium acetate buffer. During this time a voltage of 25 kV was applied. The sample is injected under 50.0 mbar, 8 s pressure.

System suitability test of the CE assay of 3-methylcamphoric acid was performed using a buffer consisting of 40 m*M* solution of sodium acetate (pH 4.0).

Detection was carried out by on-capillary UV absorbance measurement at 210 nm. All chemicals were obtained from Merck, except for naproxene (Sigma–Aldrich) and 3-methylcamphoric acid (Byk Gulden, Konstanz, Germany). The separation was carried out at a capillary oven temperature of 25°C and a potential of 25 kV.

3-Methylcamphoric acid (1.36 mg/ml) and naproxene (internal standard: 7.5 μg/ml) were dissolved in 20 mmol sodium acetate buffer adjusted to pH 4.0–methanol (87:13, v/v).

Six replicate injections (injection method) of approximately 20 μl of the solution of 3-methylcamphoric acid and naproxene were carried out. RSDs of the six injections were calculated from the quotient of peak area 3-methylcamphoric acid/peak area naproxene.

2.1.6. Thin-layer chromatography (TLC) – system performance verification/plate performance verification

A Desaga CD 60 scanning densitometer (Darmstadt, Germany), CAMAG ATS III spotter (Muttenz, Switzerland) and CAMAG twin through chamber were used.

Silica gel 60 high-performance thin-layer chromatography (HPTLC) plates (20×10) were purchased from Merck. Theophylline (Boehringer-Ingelheim,

Table 2
Routine system suitability/performance test results of robotic integrated HPLC–autosampler system[a]

SST No.	Date of SST	RSD of peak areas (%) ($n=5$)
1	12/01/98	0.3
2	12/09/98	0.4
3	12/14/98	0.6
4	06/28/99	0.2
5	06/30/99	0.3
6	07/02/99	0.3
7	07/05/99	0.3
8	07/26/99	0.2
9	07/28/99	0.5
10	08/02/99	0.1
11	08/04/99	0.1
12	09/20/99	0.3
13	09/24/99	0.3
14	09/27/99	0.4
15	10/04/99	0.3
16	10/06/99	0.3
17	10/13/99	0.2
18	10/18/99	0.3
19	10/20/99	0.2
20	10/25/99	0.3
21	10/27/99	0.2
22	11/02/99	0.3
Average		0.3

[a] For details see Experimental.

Ingelheim, Germany) was dissolved in methanol (Merck) to a concentration of approximately 10 μg/ml.

Performance testing: 16 bands (6 mm wide) of 2 μl each of the solution of theophylline were applied to the silica gel 60 HPTLC plates and developed vertically in a twin through chamber without chamber saturation using ethyl acetate as solvent. Bands were evaluated via scanning densitometry using UV at 268 nm.

RSDs were calculated from both peak areas and peak heights.

3. Results and discussion

The data presented in Tables 2–7 clearly show the broad performance variability of HPLC, GC and CE autosampler system combinations in a GMP controlled area determined by performance verification during performance qualification [15–18] and the following routine performance verification tests at specified intervals or by system suitability testing (Tables 2–6), and the variability of TLC scanning/spotting device combinations and additional plate variability. TLC plate variability is represented not only by random scatter, but also as a distinctive bias

Table 3
HPLC system (including autosampler) performance verification latest results, test interval: 6 months[a]

HPLC system	RSD of methyl ester (%) ($n=6$)	RSD of ethyl ester (%) ($n=6$)	RSD of propyl ester (%) ($n=6$)
Kontron E	0.6	0.2	1.2
Kontron G	0.4	0.6	0.6
Kontron H	0.4	0.5	0.8
Kontron J	0.2	0.2	0.4
Kontron K	0.5	0.7	1.1
Kontron L	0.7	0.6	0.7
Kontron M	0.6	0.7	1.0
Merck D6000 1	0.7	0.7	0.4
Merck D6000 2	0.6	0.7	0.6
Merck D6000 3	0.8	0.8	0.8
Merck D7000 4	0.4	0.2	0.4
Average	0.5	0.6	0.7

[a] For details see Experimental.

Table 4
Routine system suitability/performance tests of GC system direct injection[a]

SST No.	Date of SST	RSD of peak areas (%) ($n=6$)
1	02/17/98	0.5
2	03/30/98	0.5
3	05/13/98	0.4
4	07/09/98	0.7
5	01/08/99	0.9
6	04/29/99	0.7
7	06/30/99	0.7
8	07/02/99	0.7
9	10/04/99	1.0
10	11/24/99	0.5
Average		0.7

[a] For details see Experimental.

("edge-effect"). This is caused by an increase in layer thickness near the edges of the plate which results in lower peak heights and smaller peak areas on the outer tracks (tracks 1, 2 and 15, 16, Table 7).

Analytical validation is performed extensively within the pharmaceutical industry following the Guidelines of the International Conference on Harmonization (ICH) [19–21]. None of these guidelines, however, stipulates any acceptance criteria for results generated during analytical validation experiments as these will depend largely on the intended application of the procedure and the circumstances under which it will be used. An appropriate scientific rationale must be provided to justify the approach and the acceptance criteria employed.

Table 5
Routine system suitability/performance tests of GC headspace system[a]

SST No.	Date of SST	RSD of peak areas (%) ($n=6$)
1	05/05/99	0.7
2	05/11/99	0.9
3	05/26/99	1.1
4	07/19/99	0.7
5	10/04/99	1.0
6	10/19/99	0.8
7	11/04/99	2.3
8	11/10/99	0.9
9	11/12/99	0.8
10	12/13/99	1.8
Average		1.1

[a] For details see Experimental.

Table 6
Routine system suitability/performance test of a CE–autosampler system ($n=6$)[a]

SST No.	Date of SST	RSD of quotient of peak areas (%) ($n=6$)
1	08/02/99	0.6
2	09/02/99	1.2
3	23/03/99	0.7
4	26/03/99	0.2
5	29/03/99	0.9
6	01/04/99	0.9
7	28/04/99	0.7
8	20/04/99	0.3
9	07/05/99	0.5
10	06/07/99	0.8
11	07/07/99	1.1
12	09/07/99	1.0
13	07/10/99	0.5
14	06/11/99	0.7
15	08/11/99	0.6
16	09/11/99	0.6
Average		0.7

[a] For details see Experimental.

However, it has become a custom to report outstanding good precision data (either repeatability or intermediate precision) for chromatographic assays in applications, publications and/or conference lectures. This may lead to the impression, that chromatographic techniques per se are capable of controlling very tight specification limits.

But validation of a procedure is only a single event in its life cycle that may endure for more than two decades for marketed products. Precision data derived from isolated validation experiments – which may in certain cases even be selected data – must not be misinterpreted as evidence of the reliability of analytical results derived with using this procedure in later routine stages, as it does not describe true variability (uncertainty) of an analytical procedure [17,12,22] and is itself subject to day-to-day variability. The presented data from chromatographic system performance testing clearly indicate that RSDs of approximately 0.5 to 1.0% have to be taken into account for this equipment related step in the overall procedure. Table 8 shows the relation-

Table 7
TLC system/spotting device performance and plate performance testing[a]

Plate No.	Tracks evaluated (No. of tracks)	Peak height RSD (%)	Peak area RSD (%)
1	1–16 ($n=16$)	2.2	2.9
	2–15 ($n=14$)	1.8	2.5
	3–14 ($n=12$)	1.5	2.0
	5–12 ($n=8$)	1.2	1.7
2	1–16 ($n=16$)	1.9	2.4
	2–15 ($n=14$)	1.1	1.5
	3–14 ($n=12$)	0.8	1.3
	5–12 ($n=8$)	0.7	1.1
3	1–16 ($n=16$)	1.9	2.9
	2–15 ($n=14$)	1.6	2.2
	3–14 ($n=12$)	1.2	1.5
	5–12 ($n=8$)	1.1	1.6
4	1–16 ($n=16$)	1.6	2.9
	2–15 ($n=14$)	1.2	2.2
	3–14 ($n=12$)	1.2	1.8
	5–12 ($n=8$)	1.0	1.1
5	1–16 ($n=16$)	1.6	1.5
	2–15 ($n=14$)	1.3	1.3
	3–14 ($n=12$)	1.2	1.1
	5–12 ($n=8$)	1.0	0.8

[a] For details see Experimental.

ships of the different levels of variability based on our and other companies' experience [23,24].

From these data, it is possible to estimate average long-term standard uncertainties for assays using different chromatographic techniques. As the typical chromatographic assay in pharmaceutical analysis uses external standard calibration, system variability has to be considered twice:

$$\mathrm{RSD}_{\text{system total}} \sim \sqrt{\mathrm{RSD}^2_{\text{system}} + \mathrm{RSD}^2_{\text{system}}}$$

Table 9 summarizes the estimated long-term uncertainties – or procedural ruggedness – that have to be considered for different chromatographic assay techniques. Ruggedness, the long-term or site change variability as described in the USP [21,25], must clearly be differentiated from robustness, the short-term, deliberate alteration of parameters during the development of the analytical procedure to determine the optimal condition as described in the ICH Guidelines [19,20]. Unfortunately, terminology is inconsistent in literature: although both terms describe very different aspects, both are often used synonymously!

Another most important point to consider is that the estimated uncertainties as given in Table 9 are

Table 8
Estimated relations between RSDs of different stages of precision in chromatographic analytical procedures

$\mathrm{RSD}_{(\text{repeatability analytical procedure})}$[a]	$\sim 1.5 \times \mathrm{RSD}_{(\text{system performance})}$
$\mathrm{RSD}_{(\text{intermediate precision})}$[b]	$\sim 1.5 \times \mathrm{RSD}_{(\text{repeatability analytical procedure})}$
$\mathrm{RSD}_{(\text{standard uncertainty analytical procedure})}$	$\sim 1.5 \times \mathrm{RSD}_{(\text{intermediate precision})}$

[a] Repeatability = within-run precision.
[b] Intermediate precision = within-laboratory precision (same laboratory, different days, different analysts, different reagents, different equipment if possible).

Table 9
Average system performance, intermediate precision and expected estimated long-term analytical uncertainty of assays performed with different chromatographic techniques

Technique	System performance RSD (%)	Intermediate precision RSD (%)	Long-term uncertainty RSD (%)
HPLC, automated	0.4–0.5	0.6–0.8	0.9–1.1
HPLC	0.7–1.0	1.1–1.5	1.6–2.2
GC, direct injection	1.0	1.5	2.2
GC, headspace	1.6	2.3	3.5
CE	1.0	1.5	2.2
HPTLC	1.4[a]–1.9[b]	2.1–2.9	3.2–4.3

[a] Based on peak height.
[b] Based on peak area.

not the confidence intervals of a result that has to be expected during routine use of the procedure!

Given that the analytical measurement system can be represented by a normal distribution, the confidence interval of the mean ($\bar{x}$) of several independent measurements can be calculated by the equation:

$$\mathrm{ci}_{\bar{x}} = \bar{x} \pm t \cdot \left(\frac{s}{\sqrt{n}} \right)$$

t being a statistical parameter depending on the number of degrees of freedom and the probability level that may be obtained from published tables in statistical textbooks [26]. S is the standard deviation (standard uncertainty), n the number of measurements. For single determinations and a probability level of 95% – common in pharmaceutical analysis – the confidence interval of this single value can than be calculated [27,28] as follows:

$$\mathrm{ci} = \text{measured value} \pm 1.96 \cdot s$$

Therefore, for routine HPLC assays based on one single determination (injection), results have to be expected to scatter up to approximately ±4.2%. If a higher level of confidence of, say, 99% is desired, they might scatter even more, to approximately ±6.3%!

With a given true mean content of 99.5% and specification ranges of 98.5 to 100.5% [the common specification ranges for bulk drug substances (active pharmaceutical ingredients)], it is apparent that most of the HPLC procedures currently run as routine applications in the pharmaceutical industry are unlikely to be capable of controlling these specification limits.

This has been demonstrated before, using computer simulation [10,29,30] showing that HPLC assay results derived from single injections and/or sample solutions will typically not have adequate reliability and may generate up to 10% out-of-specification results – even when testing a sample of a batch with a true content that is well within specification limits.

The concept of treating injections or multiple determinations of one sample as separate results that cannot be averaged is therefore one of the most frequent and one of the most unscientifically rationalized ''out of context'' interpretations of the Barr case [9].

Chromatographic techniques are suitable for the assay of bulk drug substances (active pharmaceutical ingredients) only if multiple determinations and/or injections to be used to calculate one assay result are accepted, without evaluation of the individual injection or solution against the specifications [10,29,30]. The same applies also to impurity tests [31–33] and is even more important when chromatographic assays are part of long-term stability studies [34,35].

In other industries, analytical procedures are considered applicable if their variability contributes not more than approximately 10–20% to the overall process variability [36,37]. For pharmaceuticals and bulk drug substances (active pharmaceutical ingredients), a surprisingly high contribution of up to more than 50% of the overall variability is accepted. This may lead to OOS results that are analytical and not batch (process) related with all the subsequent, highly formalized and laborious failure investigations. Even when performing multiple replicates – a concept that for statistical and economical reasons is limited to max. 3–4 parallel tests – the confidence intervals for the mean assay results and the process variation will make it very hard to comply with the European expectation of an assay limit of 95–105% and the common 98.0–100.5% for bulk drug substances (active pharmaceutical ingredients). Approaches to calculate the risk of batch failure and to evaluate the impact of different options for changes, including the proposal to widen the specifications, have, however, been reported a few times only [2,3,38]. A very detailed strategy how to correlate the setting of specification limits, SST requirements for precision and the number of replicate determinations has been published very recently [39].

4. Conclusions

True long-term relative standard uncertainties of chromatographic procedures are higher than generally assumed. These long-term relative standard uncertainties require multiple injections and/or samples to be assayed to comply with the strict common specification limits for bulk drug substances (active

pharmaceutical ingredients) or the European expectations of 95–105% specification limits for finished pharmaceuticals [39].

For long-term stability studies and bulk drug substances (active pharmaceutical ingredients), many chromatographic assays are unlikely to be capable of detecting significant levels of change of the active content. In these applications, a combination of chromatographic purity testing and a more precise, however unspecific, assay via titration, UV measurement, etc. must be considered more appropriate [40–44].

If pharmaceuticals require chromatographic assays, as matrix effects and composition will not allow an unspecific assay to be performed, specifications should be based on a rational approach [45] – including a statement of the analytical procedure's true long-term variability – rather than accepting the ambiguous European 95–105% release and shelf life assay expectation.

Common system suitability performance parameters (e.g., RSD$\leq$2% for five or six replicate injections) are far too liberal to control performance if assays are to be performed on this equipment. An upper limit of 1.0% is proposed to allow control of the strict European specification limits or bulk drug substance (active pharmaceutical ingredients) assays.

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