WHITE PAPER

Analysis of Related Substances of Chlorhexidine Digluconate: Kinetex™ 5 µm C18 Batch Reproducibility and Effect of Gradient Delay Volume on Separation of Impurities

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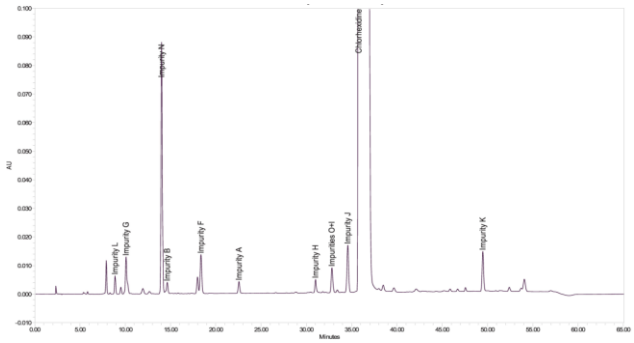
Introduction

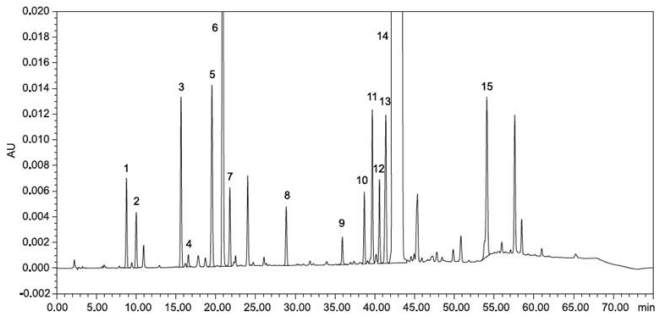
Chlorhexidine Digluconate is the gluconate salt form of Chlorhexidine, a biguanide compound used as an antiseptic agent with topical antibacterial activity.[1] Related substances include 14 known impurities among which 12 of them are specified and need to be controlled for pharmaceutical use. With an active ingredient having so many specified impurities it is usually a challenge to maintain the separation of all impurities in a controlled and repeatable manner. The current valid Ph. Eur. monograph for related substances of Chlorhexidine Digluconate solution has a few drawbacks. Figure 1 represents the chromatogram of the reference standard Chlorhexidine for system suitability obtained with the current valid Ph. Eur. monograph, indicating a poor separation of impurities N and B (retention time approximately 14 min), and a co-elution of impurities O and I (retention time approximately 33 min). Additionally, the analytical method using a Luna™ C18(2) column, as currently suggested, is very batch sensitive and dependent on the gradient delay volume of the LC System used. Co-elution of some impurity peaks, and in some cases even a reversal of peak order, has been reported which makes quality control of this pharmaceutical drug problematic in routine analysis. The currently required system suitability criteria (a minimum resolution of 3.0 between peaks due to impurities L and G) is not sufficient and unfortunately easily allows mistakes to be made in system suitability evaluation prior to sample analysis.

Figure 1. Chromatogram of Reference Standard Chlorhexidine for System Suitability CRS Batch 4.[2]



The latest draft revision of the Ph. Eur. monograph for related substances of Chlorhexidine Digluconate describes an optimized analytical procedure for related substances with a better control of impurities and a significant improvement of peak separation (Figure 2).[3] The system suitability criteria has been updated to consider a more critical separation of impurities. The revision proposes the use of an end-capped solid core octadecylsilyl silica gel stationary phase and an optimized gradient elution. The initial isocratic step at 100 % mobile phase A is increased from 2 min to 5 min, which results in a more robust gradient elution when switching between different LC instruments and enables smooth method verification. Retention time of Chlorhexidine Digluconate is hence increased from 35 min to 42 min. A gradient delay volume (D) of 1.14 mL was reported for this draft, suggesting that a low-pressure gradient or a quaternary pump was used for method development. Today, many QC laboratories have binary LC systems with much lower gradient delay volumes and it is often necessary to adjust the gradient time points to account for differences in delay volumes.

Figure 2. Chromatogram for the Test for Related Substances: Solution of Chlorhexidine Spiked with Impurities A, B, E, F, G, H, I, J, K, L, M, N, O, and Q.[3]



Peak No.	Substance
1	Impurity E
2	Impurity L
3	Impurity G
4	Impurity Q
5	Impurity F
6	Impurity N
7	Impurity B
8	Impurity A
9	Impurity M
10	Impurity H
11	Impurity O
12	Impurity I
13	Impurity J
14	Chlorhexidine
15	Impurity K

Batch-to-batch reproducibility with a representative pharmaceutical system suitability standard is an important QC parameter in a method validation procedure. The influence of a gradient delay volume on the resolution should be negligible for a robust LC method. This study evaluates the performance of six different batches of Kinetex™ 5 µm C18 columns, which is the proposed column for the new Ph. Eur. monograph for related substances of Chlorhexidine Digluconate.[3] The influence of the gradient delay volume on resolution was evaluated using a single column batch on different LC systems with delay volumes ranging from 0.80 mL to 1.36 mL. The gradient delay volumes were experimentally determined using the procedure described in the Ph.Eur. monograph for chromatographic separation techniques / Dwell volume.[4]

Materials and Methods

The study was undertaken in a GMP certified laboratory using LC quality reagents. All the reference solutions were prepared as indicated in Ph. Eur. monograph for Chlorhexidine Digluconate. Preparation of standard solutions is described in **Table 1**. The following certified reference standards (CRS) were purchased from the European Directorate for the Quality of Medicines & HealthCare (EDQM) – Council of Europe; Postal address: 7 Allee Kastner CS 30026 F - 67081 Strasbourg (France):

- C1510000, Chlorhexidine CRS
- Y0001545, Chlorhexidine for System Suitability CRS

Table 1. Preparation of Solutions.

Solution	Preparation	Concentration of Chlorhexidine
Test Solution	Dissolve 4 mg of *Chlorhexidine CRS* in 2.0 mL of Mobile Phase A.	2 mg/mL
Reference solution (a)	Dissolve 10 mg of *Chlorhexidine for System Suitability CRS* in 2.0 mL of Mobile Phase A.	not available
Reference Solution (b)	Transfer 0.5 mL of Test Solution into a flask and dilute to 50.0 mL with Mobile Phase A.	0.02 mg/mL
Solution for System Sensitivity (SSens)	Transfer 1.0 mL of Reference Solution (b) into a flask and dilute to 20.0 mL with Mobile Phase A.	0.001 mg/mL (disregard limit, 0.05%)

System sensitivity was tested at the reporting threshold of 0.05 % by preparing a standard solution of Chlorhexidine at concentration of 0.001 mg/mL (SSens). This solution was injected in a single replicate and signal-to-noise (S/N) ratio of Chlorhexidine peak was calculated. A criteria S/N ≥ 10 indicates that the quantification level is at least equal to the reporting threshold, which is required in QC methods for related substances.

Retention time and tailing factor of the Chlorhexidine peak were evaluated by injecting a standard solution of Chlorhexidine at concentration of 0.02 mg/mL (Reference Solution (b)). This solution is usually used for calibration purposes and system repeatability in routine analysis for related substances and is prepared at approximately 1 % relative to the working concentration of Chlorhexidine in the Test Solution.

Reference solution (a) containing Chlorhexidine and all its known specified impurities (Chlorhexidine for System Suitability CRS) was used to identify the impurities by comparing relative retention times reported in the monograph draft (**Figure 2**) and to evaluate system suitability. System suitability requirements are a minimum resolution of 3.0 between peaks due to impurities L and G, and minimum peak-to-valley ratio of 2.0, where H_p = height above the baseline of the peak due to impurity B and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity N. This solution was injected 6 times in succession to determine the %RSDs of peak areas and retention times for Chlorhexidine and all impurities. These results were used for batch-to-batch reproducibility evaluation.

A sample of Chlorhexidine Digluconate solution (200 g/L) was not available. Instead, Chlorhexidine CRS was used for preparation of a Test Solution at concentration of 2 mg/mL, which corresponds to the prescribed working concentration of Chlorhexidine in the Test Solution. It was injected in a single replicate to evaluate the separation of impurities in a "real sample."

All reference standard solutions were prepared freshly for each column test and stored at 8 °C immediately after preparation. The stability of Reference Solution (a) was tested by storing the solution in a clear vial on a laboratory benchtop at room temperature and injecting it at different time intervals over a period of 67 hours. All solutions were injected into an equilibrated LC system, which was achieved after several injections of solvent and running the gradient elution.

A single column batch was used for evaluating the influence of gradient delay volume on the separation of all 14 impurities of Chlorhexidine. The LC system used for testing batch-to-batch reproducibility has a gradient delay volume of 1.16 mL (Agilent® 1260 Infinity II system). Further tests were performed on a Shimadzu® Nexera® XR system applying a low-pressure/quaternary pump (D = 1.36 mL) and a high-pressure gradient/binary pump (D = 0.80 mL) and injecting Reference Solution (a).

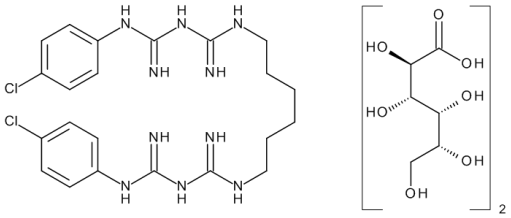
LC Conditions

Column: Kinetex 5 µm C18
Dimensions: 250 x 4.6 mm
Part No.: 00G-4601-E0
Mobile Phase: A: 0.1 % Trifluoroacetic Acid in Acetonitrile / 0.1 % Trifluoroacetic Acid in Water (15:85, v/v)
B: 0.1 % Trifluoroacetic Acid in Acetonitrile / 0.1 % Trifluoroacetic Acid in Water (90:10, v/v)
Gradient:

Time (min)	%B	
0	0	
5	0	
45	26.7	
50	26.7	
55	34.7	
65	34.7	
66	0	} Column equilibration
72	0	

Flow Rate: 1 mL/min
Injection Volume: 10 µL
Temperature: 30 °C
LC System: Agilent 1260 Infinity, D = 1.16 mL
Shimadzu Nexera XR, low pressure quaternary pump, D = 1.36 mL
Shimadzu Nexera XR, high pressure binary pump, D = 0.80 mL
Detection: UV @ 254 nm, 20 Hz acquisition rate

Figure 3. Chlorhexidine Digluconate Structure.



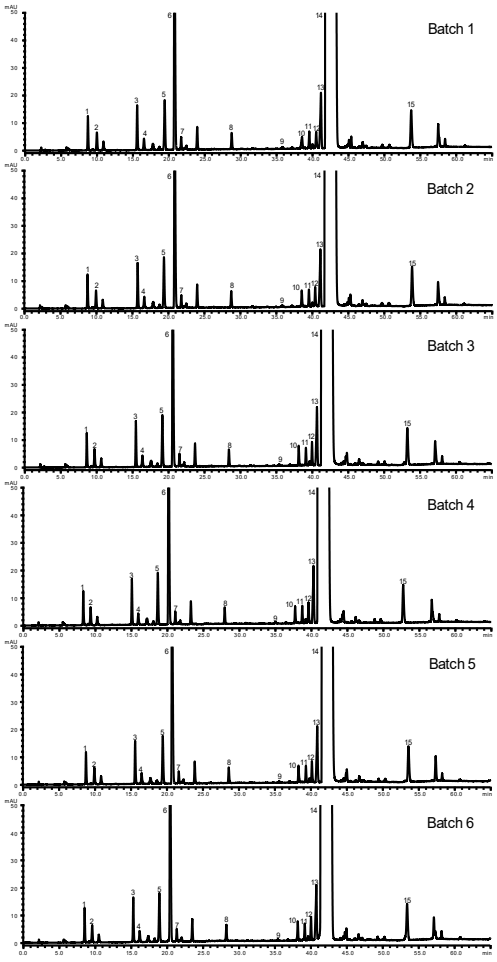
Results and Discussion

The system sensitivity, applying all six column batches, was easily achieved at the concentration level of reporting threshold (0.05 %) with values of S/N ratio for the peak of Chlorhexidine ranging from 350 to 440. Such high values of S/N indicate that the working concentration of Chlorhexidine in the Test Solution could be decreased to at least 1 mg/mL. This means a lower sample load onto the column and a single method optimization parameter.

The retention time and tailing factor for the Chlorhexidine peak at the concentration of 0.02 mg/mL ranged between 42.251 min and 43.277 min, and from 1.3 to 1.5 for all six batches of columns, respectively. Tailing factor value for the peak used for quantification should be between 0.8 and 1.8.

Chromatograms of Reference Solution (a) obtained on each batch are shown in **Figure 4**. In all cases the peaks of Impurity N and Impurity B were baseline separated thus exceeded the system suitability criteria of obtaining a minimum peak-to-valley ratio value of 2.0. Hence, the resolution factor between these peaks was calculated and ranged between 4.1 and 4.3. Such a resolution factor indicates that the peaks are well separated and small changes in chromatographic conditions should not influence their separation, resulting in a robust method. The separation of peaks of Impurity O and Impurity I was also significantly improved compared to the separation shown in **Figure 1**. Overall, all 14 known impurities were baseline separated and retention times repeatable between six successive injections (**Table 2**). The elution of unknown peaks at retention time of approximately 45 min and 57 min somewhat differ among different batches of columns but they do not belong to any known impurities and are disregarded in the quantification method.

Figure 4. Chromatograms of Reference Solution (a) on Six Different Batches of Kinetex™ 5 µm C18 Columns.



Peak No.	Substance
1	Impurity E
2	Impurity L
3	Impurity G
4	Impurity Q
5	Impurity F
6	Impurity N
7	Impurity B
8	Impurity A
9	Impurity M
10	Impurity H
11	Impurity O
12	Impurity I
13	Impurity J
14	Chlorhexidine
15	Impurity K

Table 2. Average Retention Times of Chlorhexidine and its Impurities; Average Resolution Factor (R) Between Peaks Due to Impurity N and B.

Peak No.	Analyte	Retention Time (min)					
		Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6
1	Impurity E	8.76	8.79	8.64	8.34	8.76	8.54
2	Impurity L	10.00	9.97	9.73	9.36	9.93	9.58
3	Impurity G	15.62	15.76	15.49	15.10	15.61	15.31
4	Impurity Q	16.57	16.68	16.40	16.00	16.51	16.19
5	Impurity F	19.44	19.41	19.17	18.70	19.43	18.93
6	Impurity N	20.80	20.89	20.60	20.19	20.73	20.42
7	Impurity B	21.74	21.83	21.55	21.14	21.67	21.36
8	Impurity A	28.77	28.77	28.44	28.00	28.62	28.27
9	Impurity M	35.83	35.86	35.45	35.08	35.62	35.45
10	Impurity H	38.57	38.57	38.16	37.81	38.32	38.21
11	Impurity O	39.57	39.57	39.16	38.79	39.34	39.19
12	Impurity I	40.52	40.43	40.01	39.64	40.17	40.06
13	Impurity J	41.19	41.16	40.70	40.35	40.92	40.80
14	Chlorhexidine	41.86	41.83	41.37	40.99	41.65	41.46
15	Impurity K	53.76	53.92	53.31	52.84	53.61	53.45
		Resolution					
6/7	$R_{Impurity\ N/Impurity\ B}$	4.136	4.099	4.270	4.275	4.119	4.261
N = 6 Replicates							

The repeatability of the system was evaluated by 6 successive injections of Reference Solution (a) and resulted in %RSD of retention times below 0.1 % for all peaks (**Table 3**) and %RSD of peak area ranging between 0.1 % and 3.0 % (**Table 4**). The %RSD value for the Chlorhexidine peak area, although at a rather high concentration, was below 0.2 % indicating a very precise system.

Table 3. Retention Time %RSDs for Chlorhexidine and its Impurities.

Peak No.	Analyte	%RSD					
		Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6
1	Impurity E	0.040	0.059	0.077	0.076	0.043	0.030
2	Impurity L	0.048	0.018	0.089	0.032	0.054	0.073
3	Impurity G	0.081	0.101	0.091	0.088	0.114	0.015
4	Impurity Q	0.075	0.094	0.078	0.077	0.105	0.010
5	Impurity F	0.039	0.030	0.046	0.022	0.058	0.047
6	Impurity N	0.056	0.087	0.060	0.073	0.076	0.016
7	Impurity B	0.053	0.078	0.058	0.069	0.064	0.015
8	Impurity A	0.030	0.038	0.030	0.058	0.026	0.015
9	Impurity M	0.018	0.041	0.049	0.067	0.038	0.038
10	Impurity H	0.036	0.037	0.049	0.078	0.038	0.041
11	Impurity O	0.016	0.032	0.048	0.064	0.033	0.036
12	Impurity I	0.023	0.019	0.042	0.048	0.026	0.033
13	Impurity J	0.020	0.026	0.045	0.055	0.029	0.036
14	Chlorhexidine	0.021	0.031	0.049	0.058	0.029	0.037
15	Impurity K	0.016	0.043	0.073	0.098	0.040	0.059
N = 6 Replicates							

Table 4. Peak Area %RSDs for Chlorhexidine and its Impurities.

Peak No.	Analyte	%RSDs					
		Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6
1	Impurity E	0.149	0.263	0.120	0.089	0.121	1.120
2	Impurity L	0.074	0.132	0.270	0.317	0.209	0.182
3	Impurity G	0.205	0.270	0.164	0.109	0.153	0.057
4	Impurity Q	0.270	0.339	0.349	0.105	0.314	0.406
5	Impurity F	0.093	0.182	0.179	0.076	0.106	0.135
6	Impurity N	0.142	0.247	0.194	0.108	0.161	0.081
7	Impurity B	0.850	0.765	0.802	0.666	0.643	0.998
8	Impurity A	0.111	0.410	0.233	0.077	0.100	0.118
9	Impurity M	1.363	1.502	1.010	1.521	2.138	0.732
10	Impurity H	1.045	1.625	0.636	3.001	0.403	0.354
11	Impurity O	1.560	1.952	0.683	0.179	0.589	0.277
12	Impurity I	1.886	1.058	1.184	2.349	1.078	0.178
13	Impurity J	0.452	0.088	0.222	0.136	0.248	0.131
14	Chlorhexidine	0.150	0.238	0.168	0.130	0.153	0.070
15	Impurity K	0.513	1.133	0.385	0.304	0.301	0.582
N = 6 Replicates							

Chromatograms of the Test Solution at concentration of 2 mg/mL Chlorhexidine obtained on all six batches of columns are shown in **Figure 5**. Chlorhexidine impurities B, A, H, O, I and K were above reporting threshold, easily identified and very well separated.

Reference Solution (a) proved to be stable for its purpose of use when stored for 67 hours at room temperature. An increase of peak area of impurities B (21.6 min) and K (53.4 min) was observed (**Figure 6**).

Figure 5. Comparison of Chromatograms of the Test Solution Obtained on Six Different Batches of Kinetex™ 5 µm C18 Columns.

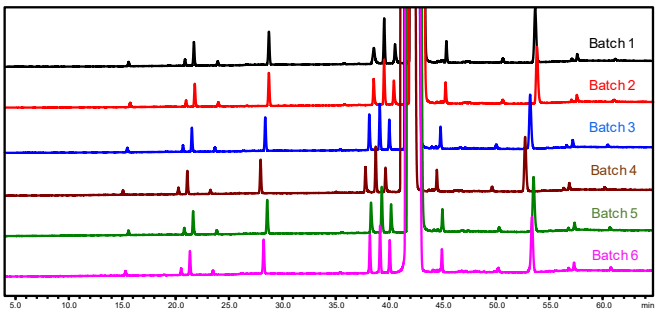
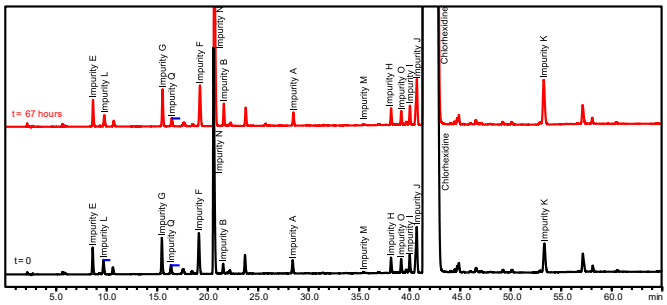


Figure 6. Stability of Reference Solution (a), Initial (t=0) Chromatogram and After 67 Hours of Storage in a Clear Vial on a Benchtop at Room Temperature.



Column Batch 3 was used for testing the influence of gradient delay volume on the separation of Chlorhexidine impurities. **Table 5** and **Figure 7** represent the retention times of Chlorhexidine and its impurities obtained with different LC systems. The retention times and the resolution factor between peaks of impurity N and B are very well comparable among each other indicating that the method is robust and that the gradient delay volume has no significant influence on the separation of impurities.

The method may be applied on low pressure and high-pressure gradient LC systems without any gradient modifications being necessary enabling an easy method transfer between different laboratories. The gradient adjustment per Ph. Eur. 2.2.46[4] was however considered (**Table 6**) but had no impact on the results since the difference in gradient delay volumes did not impact retention times, resolution, or overall separation. Thus, the results and data are not shown.

Table 5. Retention Times of Chlorhexidine and its Impurities Obtained on Column Batch 3 Using Different LC Systems with Different Gradient Delay Volumes.

Peak No.	Analyte	%RSD		
		Agilent® 1260 D = 1.16 mL	Nexera® XR - LPG D = 1.36 mL	Nexera XR - HPG D = 0.80 mL
1	Impurity E	8.64	8.56	8.55
2	Impurity L	9.73	9.63	9.61
3	Impurity G	15.49	15.30	15.05
4	Impurity Q	16.40	16.20	15.94
5	Impurity F	19.17	19.09	18.94
6	Impurity N	20.60	20.43	20.20
7	Impurity B	21.55	21.39	21.17
8	Impurity A	28.44	28.49	28.30
9	Impurity M	35.45	34.63	35.46
10	Impurity H	38.16	38.41	38.24
11	Impurity O	39.16	39.39	39.23
12	Impurity I	40.01	40.27	40.11
13	Impurity J	40.70	40.98	40.82
14	Chlorhexidine	41.37	41.64	41.47
15	Impurity K	53.31	53.57	53.74
Resolution				
6/7	$R_{impurity\ N/impurity\ B}$	4.136	4.099	4.270

Figure 7. Comparison of Chromatograms of Reference Solution (a) Obtained on Column Batch 3 Using Different LC Systems with Different Gradient Delay Volumes (See Retention Times in **Table 5**).

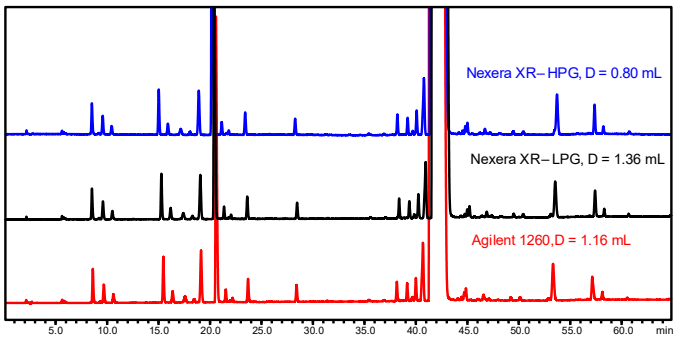


Table 6. Adjusted Time Points Due to Differences in Gradient Delay Volumes. Calculations were made according to Ph. Eur. Monograph for Chromatographic Separation Techniques / Adjustments of Chromatographic Conditions / Gradient Elution / Dwell Volume.[4]
Note: Gradient Delay Volume Stated in the Monograph Draft Reported as 1.14 mL.

Agilent® 1260 Infinity II, D = 1.16 mL		
Time (min)	**%A**	**%B**
0	100	0
5	100	0
45	73.3	26.7
50	73.3	26.7
55	65.3	34.7
65	65.3	34.7

Shimadzu® Nexera® XR – LPG, D = 1.36 mL		
Time (min)	**%A**	**%B**
0	100	0
4.78	100	0
44.78	73.3	26.7
49.78	73.3	26.7
54.78	65.3	34.7
64.78	65.3	34.7

Shimadzu Nexera XR – HPG, D = 0.80 mL		
Time (min)	**%A**	**%B**
0	100	0
5.34	100	0
45.34	73.3	26.7
50.34	73.3	26.7
55.34	65.3	34.7
65.34	65.3	34.7

Conclusion

The new proposed method in the latest draft revision of the Ph.Eur. monograph for related substances of Chlorhexidine Digluconate suggests improved control of impurities and a different, more critical, system suitability criteria. This study also demonstrates the batch-to-batch reproducibility for Kinetex 5 µm C18 columns as proposed in the draft. The reproducibility was evaluated using a representative pharmaceutical QC suitability mixture containing 12 specified impurities. System suitability requires a minimum value of 2.0 for peak-to-valley ratio of peaks due to impurity N and impurity B, which was exceeded as baseline separation with resolution factor ranging between 4.1 and 4.3 was observed on all six column batches tested. System sensitivity was easily achieved at a concentration of reporting threshold of 0.05 % with S/N values for the Chlorhexidine peak ranging from 350 to 440 (criteria S/N ≥ 10 for limit of quantification). The injections were highly repeatable (%RSD values of retention times < 0.1 %) with a negligible difference in retention times for all peaks across the six different batches of columns used. The separation for peaks of Impurity O and Impurity I was also significantly improved.

Additionally, the Reference Solution (a) was shown to be stable for purpose of use (identification of specified impurities and system suitability evaluation) for at least 67 hours stored at room temperature in a clear vial.

Results obtained on different column batches and different LC systems, with gradient delay volumes ranging from 0.80 mL to 1.36 mL, demonstrated that the proposed method is robust, which is an important information for a method verification with a gradient elution. Adjustments to the gradient times should be made when systems with different gradient delay volumes or dwell volumes are used. In this method, we did not observe that this was necessary. The inclusion of a 5-minute isocratic hold at the beginning of the chromatographic run minimizes the differences in gradient delay volumes when using different systems, thus avoiding the potential for errors when transferring this method across different systems.

In this case, as the difference in gradient delay volumes is small relative to the column volume, and the gradient is shallow, the volumes are likely to be minor contributory factors to the overall separation.

References

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3. Pharmaeuropa 35.4: PA/PH/Exp. 10 B/T (23) 65 ANP, Chlorhexidine digluconate solution, XXXX:0658
4. Ph. Eur. 11.0, 01/2023:20246, Chromatographic separation techniques

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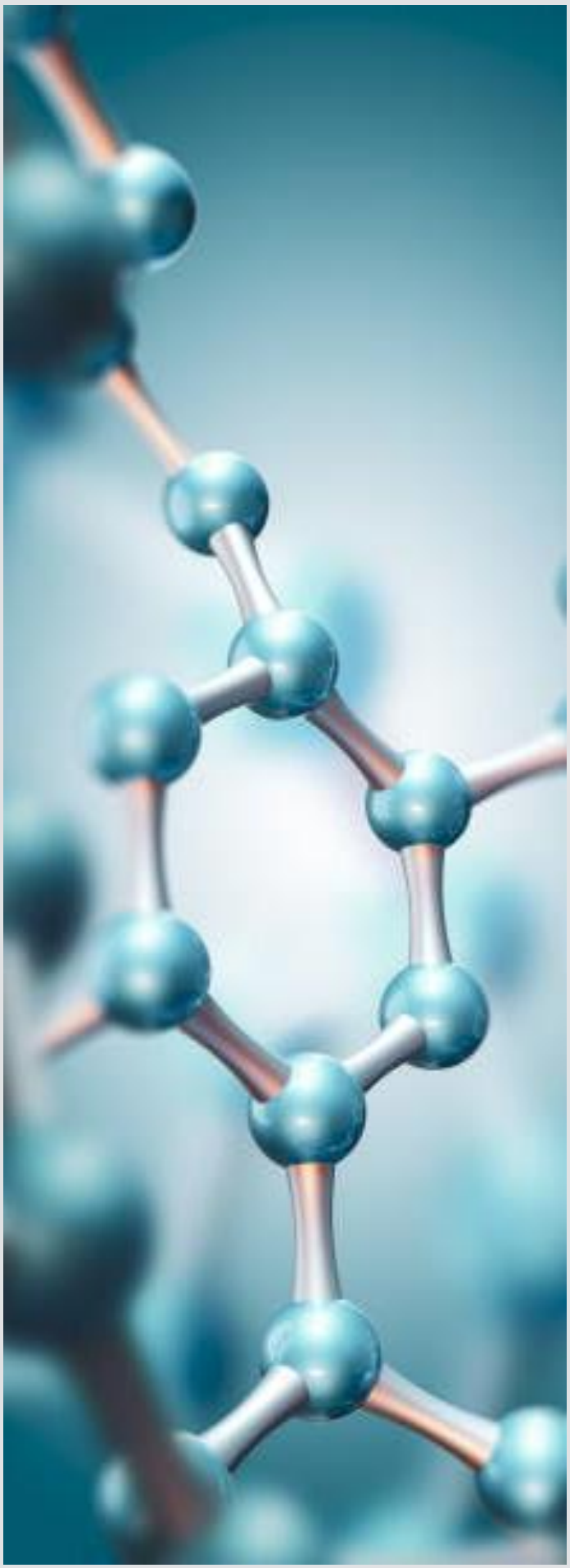

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